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(54) Title: VACCINE

(57) Abstract: The invention relates to polynucleotides for DNA vaccination which polynucleotides encode an HIV envelope protein or fragment or immunogenic derivative fused to an additional HIV protein selected from a non-structural protein or capsid protein or fragment or immunogenic derivative thereof. Preferably the HIV envelope molecule is gp120 and preferred fusions include one or more of HIV Nef, Gag, RT or Tat. Preferably the HIV envelope molecule is non-glycosylated in mammalian cells.

Vaccine**Field of the Invention**

5 This invention relates to nucleic acid constructs, vectors comprising such constructs, methods of preparing the vectors and constructs and their use in prophylaxis or therapy, in particular therapeutic vaccines. The invention further relates to host cells comprising the constructs and vectors and to polypeptides encoded by the constructs as well as to the polypeptides per se. The invention further relates to pharmaceutical
10 formulations comprising the constructs and vectors and to the use of the constructs and vectors in medicine. The invention relates in particular to DNA vaccines that are useful in the prophylaxis and treatment of HIV infections, more particularly when administered by particle mediated delivery.

15 Background to the Invention

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts
20 thus far have not been successful.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger precursor molecule (gp160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.
25

30 The gp120 protein is the principal target of neutralizing antibodies, but unfortunately the most immunogenic regions of the proteins (V3 loop) are also the most variable parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine

antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly protective vaccine. The gp120 protein does also contain epitopes that are recognized by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism.

5 In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 may be considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

10 Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the gag and pol genes and other non-structural proteins such as Rev, Nef, Vif and Tat (Green et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., 11, 5, 390 et seq (1992)).

15 HIV Tat and Nef proteins are early proteins, that is they are expressed early in infection and in the absence of structural protein.

The Nef protein is known to cause the removal of CD4, the HIV receptor, from the cell surface, but the biological importance of this function is debated. Additionally Nef interacts with the signal pathway of T cells and induces an active state, which in turn may promote more efficient gene expression. Some HIV isolates have mutations in this region, which cause them not to encode functional protein and are severely compromised in their replication and pathogenesis *in vivo*.

20 The Tat gene gives rise to a number of differentially spliced transcripts at different times during infection. The first exon encodes an 86 amino acid protein which dominates early in infection. The second exon encodes an additional 14 amino acids, and this partially spliced form of Tat is found late in infection. Both forms are fully functional transactivators, but the longer form also contains an RGD motif important for binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Tat binds to a short-stem loop structure, known as the transactivation response element (TAR), that is located at the 5' terminus of

HIV RNAs, and up-regulates transcription from the HIV LTR at least 1000-fold. Tat has a role in promoting the elongation phase of HIV infection and stimulates the production of full-length viral transcripts. Tat can affect the expression of a number of cellular genes and can activate the expression of a number of cellular genes including

5 TNF, IL-2 and IL-6, and regulates expression of p53 and Bcl-2. Tat is produced in excess and is secreted from infected cells. This extra-cellular Tat can enter other cells and may prime cells for infection by HIV or accelerate the rate of HIV replication in newly infected cells.

10 In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12th Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and one dose recombinant protein). The results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

15

The Gag gene is translated from the full-length RNA to yield a precursor polyprotein which is subsequently cleaved into 3 – 5 capsid proteins; the matrix protein, capsid protein and nucleic acid binding protein and protease. (1. Fundamental Virology, Fields BN, Knipe DM and Howley M 1996 2. Fields Virology vol 2 1996).

The gag gene gives rise to the 55-kilodalton (kD) Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. During translation, the N terminus of p55 is myristoylated, triggering its association with the cytoplasmic aspect of cell membranes. The membrane-associated Gag polyprotein recruits two copies of the viral genomic RNA along with other viral and cellular proteins that triggers the budding of the viral particle from the surface of an infected cell. After budding, p55 is cleaved by the virally encoded protease (a product of the pol gene) during the process of viral maturation into four smaller proteins designated MA (matrix [p17]), CA (capsid [p24]), NC (nucleocapsid [p9]), and p6.(4).

In addition to the 3 major Gag proteins, all Gag precursors contain several other regions, which are cleaved out and remain in the virion as peptides of various sizes. These proteins have different roles e.g. the p2 protein has a proposed role in regulating activity of the protease and contributes to the correct timing of proteolytic processing.

5

The MA polypeptide is derived from the N-terminal, myristoylated end of p55. Most MA molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle. A subset of MA is recruited inside the deeper layers of the virion where it becomes part of the complex which escorts the viral DNA to the 10 nucleus. These MA molecules facilitate the nuclear transport of the viral genome because a karyophilic signal on MA is recognized by the cellular nuclear import machinery. This phenomenon allows HIV to infect non-dividing cells, an unusual property for a retrovirus.

15 The p24 (CA) protein forms the conical core of viral particles. Cyclophilin A has been demonstrated to interact with the p24 region of p55 leading to its incorporation into HIV particles. The interaction between Gag and cyclophilin A is essential because the disruption of this interaction by cyclosporin A inhibits viral replication.

20 The NC region of Gag is responsible for specifically recognizing the so-called packaging signal of HIV. The packaging signal consists of four stem loop structures located near the 5' end of the viral RNA, and is sufficient to mediate the incorporation of a heterologous RNA into HIV-1 virions. NC binds to the packaging signal through interactions mediated by two zinc-finger motifs. NC also facilitates reverse 25 transcription.

The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions. The p6 region also contains a so-called late domain which is required for the efficient release 30 of budding virions from an infected cell.

The Pol gene encodes two proteins containing the two activities needed by the virus in early infection, the RT and the integrase protein needed for integration of viral DNA into cell DNA. The primary product of Pol is cleaved by the virion protease to yield the amino terminal RT peptide which contains activities necessary for DNA synthesis

5 (RNA and DNA directed DNA polymerase, ribonuclease H) and carboxy terminal integrase protein. HIV RT is a heterodimer of full-length RT (p66) and a cleavage product (p51) lacking the carboxy terminal Rnase integrase domain.

RT is one of the most highly conserved proteins encoded by the retroviral genome.

10 Two major activities of RT are the DNA Pol and Ribonuclease H. The DNA Pol activity of RT uses RNA and DNA as templates interchangeably and like all DNA polymerases known is unable to initiate DNA synthesis de novo, but requires a pre existing molecule to serve as a primer (RNA).

15 The Rnase H activity inherent in all RT proteins plays the essential role early in replication of removing the RNA genome as DNA synthesis proceeds. It selectively degrades the RNA from all RNA - DNA hybrid molecules. Structurally the polymerase and ribo H occupy separate, non-overlapping domains with the Pol covering the amino two thirds of the Pol.

20 The p66 catalytic subunit is folded into 5 distinct subdomains. The amino terminal 23 of these have the portion with RT activity. Carboxy terminal to these is the Rnase H Domain.

25 After infection of the host cell, the retroviral RNA genome is copied into linear ds DNA by the reverse transcriptase that is present in the infecting particle. The integrase (reviewed in Skalka AM '99 Adv in Virus Res 52 271-273) recognises the ends of the viral DNA, trims them and accompanies the viral DNA to a host chromosomal site to catalyse integration. Many sites in the host DNA can be targets

30 for integration. Although the integrase is sufficient to catalyse integration in vitro, it is not the only protein associated with the viral DNA in vivo - the large protein - viral DNA complex isolated from the infected cells has been denoted the pre integration

complex. This facilitates the acquisition of the host cell genes by progeny viral genomes.

The integrase is made up of 3 distinct domains, the N terminal domain, the catalytic
5 core and the C terminal domain. The catalytic core domain contains all of the requirements for the chemistry of polynucleotidyl transfer.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong promoter, the gene of interest which encodes an antigenic peptide and a
10 polyadenylation/transcriptional termination sequence. The gene of interest may encode a full protein or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which it is intended to protect against. The plasmid can be grown in bacteria, such as for example *E.coli* and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before
15 being administered to the host. Following administration the plasmid is taken up by cells of the host, or delivered directly into the host cells, where the encoded peptide is produced. The plasmid vector will preferably be made without an origin of replication functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned.

20

There are a number of advantages of DNA vaccination relative to traditional vaccination techniques. First, it is predicted that because the proteins that are encoded by the DNA sequence are synthesised in the host, the structure or conformation of the protein will be similar to the native protein associated with the disease state. It is also
25 likely that DNA vaccination will offer protection against different strains of a virus, by generating a cytotoxic T lymphocyte response that recognises epitopes from conserved proteins. The technology also offers the possibility of combining diverse immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.

30

Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

5 Doe et al (1994) Eur J Immunol, 24: 2369-2376 investigated how variations in glycosylation affected the CD8+ CTL response to gp120 and found that gp120 produced in mammalian CHO cells had a reduced ability to prime CTL responses when compared with insect or yeast cell-derived envelope proteins unless N-linked oligosaccharides were removed prior to immunization.

10

It has now been discovered that there are benefits to be gained by employing a polynucleotide encoding a non-glycosylated HIV envelope protein in a vaccine for HIV. Surprisingly, a DNA vector expressing gp120 without a secretion signal and which is thus not glycosylated or secreted from the cell is a more effective stimulator
15 of CTL responses than a DNA vector expressing gp120 with its native secretion signal. Since the secretion signal is responsible for directing the gp120 to the intracellular site where glycosylation takes place, gp120 which lacks its native secretion signal is not glycosylated. Moreover, with the presence of a non-structural HIV protein such as tat in a fusion protein with the non-glycosylated gp120, CTL
20 responses to the gp120 are augmented. In contrast, Tat in a fusion protein with normal gp120 prevents secretion but does not result in an augmented immune response. The non-glycosylated gp120 can also be successfully expressed in a fusion protein with other HIV antigens, both structural and non-structural.

25 **Summary of the Invention**

The present invention therefore provides novel constructs for use in nucleic acid or polypeptide vaccines for the prophylaxis and treatment of HIV infections and AIDS.

30 In one aspect the invention provides a polynucleotide which comprises a sequence encoding an HIV envelope protein or fragment or immunogenic derivative thereof, fused to at least one sequence encoding an HIV non-structural or capsid protein or

fragment or immunogenic derivative thereof, operably linked to a heterologous promoter.

Preferably the HIV envelope protein is gp120. Alternatively it may be other forms of
5 the envelope protein such as gp160 or gp140.

Preferably the at least one HIV non-structural protein is selected from one or more of Nef, RT or Tat or fragments or immunogenic derivatives thereof. Optionally a structural protein, particularly Gag, may be further included in the fusion.

10

Alternatively the at least one HIV protein to which the envelope is fused may be a capsid protein such as Gag or a fragment or immunogenic derivative thereof.

15

In one embodiment the fusion protein is a gp120 and RT-containing fusion protein, optionally also comprising Gag and/or Nef.

In another embodiment the fusion protein is a gp120 and Gag-containing fusion protein optionally also comprising RT and/or Nef.

20

In a further embodiment the fusion protein is a gp120 and Nef-containing fusion protein optionally also comprising RT and/or Gag.

In the following preferred embodiments the fusion protein is a fusion of gp120, RT, Nef and Gag or fragments or immunogenic derivatives thereof:

25

gp120-RT-Nef-Gag

RT-Nef-Gag-gp120

30

Another embodiment is a fusion comprising gp120 and Tat or fragments or immunogenic derivatives thereof. In such an embodiment the polynucleotide according to the invention comprises a gp120 encoding sequence linked to a Tat encoding sequence to encode a gp120 and Tat-containing fusion protein.

In a particular embodiment the gp120 and Tat sequence is further linked to a Nef encoding sequence to encode a gp120, Tat and Nef-containing fusion protein, most preferably a gp120-Nef-Tat fusion.

5 Additional HIV sequences may be included such as a Gag encoding sequence.

In another particular embodiment the fusion encoded by the polynucleotide according to the invention is a gp120-Gag-Nef-Tat fusion.

10 Preferably the Tat sequence for use in the invention is mutated so that it encodes a biologically inactive Tat which lacks transactivation activity but which maintains its immunogenic epitopes.

Tat transactivation activity can be measured for example by an HIV LTR reporter system such as a CAT assay system in which the chloramphenicol-acetyl transferase reporter gene is under the control of the long terminal repeat of HIV-1. One specific CAT assay which is suitable uses the HL3T1 cell line which is described in Felber and Pavlakis (1988) Science 239: 184-187 and also used in Mischiati et al (2001) Antisense Nucleic Acid Drug Dev Aug 11 (4): 209-17. HL3T1 is a HeLa cell line 20 which contains stably integrated silent copies of HIV-1 LTR promoter linked to the CAT gene. These cells are transfected with DNA vectors containing the Tat gene, modified or not. CAT is produced upon presence of an active Tat and measured by ELISA.

25 One preferred mutated Tat sequence (originating from BH10 molecular clone) bears mutations in the active site region (Lys41→Ala) and in the RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

30 Optionally the Nef sequence for use in the invention is truncated to remove the sequence encoding the N terminal region i.e. removal of 30-85, preferably 60-85, preferably the N terminal 65 amino acids (the latter truncation is referred to herein as trNef). Advantageously the Nef may be modified to remove one or more

myristylation sites. For example the Gly 2 myristylation site may be removed by deletion or substitution. Alternatively or additionally the Nef may be modified to alter the dileucine motif of Leu 174 and Leu 175 by deletion or substitution of one or both leucines. The importance of the dileucine motif in CD4 downregulation is described
5 e.g. in Bresnahan P.A. et al (1998) Current Biology, 8(22): 1235-8.

The RT polynucleotide for use in the invention preferably encodes a mutation to substantially inactivate any reverse transcription activity. A preferred inactive mutant involves the substitution of W tryptophan 229 for K lysine. See WO 03/025003.

10

Preferably the Gag for use in the invention does not encode the Gag P6 polypeptide. Preferred Gag sequences for use in the invention comprise P17 and/or 24.

15

Preferably one or more of the HIV sequences included in the polynucleotide according to the invention encoding e.g. gp120, Nef, Tat, Gag or RT is or are codon optimised for mammalian cells, most preferably such that it/they resemble a highly expressed human gene in their codon use.

20

The fusion may contain further HIV sequences. It will be understood that for all of the HIV sequences included in the invention, these do not necessarily represent sequences encoding the full length or native proteins. Immunogenic derivatives such as truncated or otherwise altered e.g. mutated proteins are also contemplated, as are fragments which encode at least one HIV epitope, preferably a CTL epitope, typically a peptide of at least 8 amino acids. Polynucleotides which encode a fragment of at least 8, for example 8-10 amino acids or up to 20, 50, 60, 70, 100, 150 or 200 amino acids in length are considered to fall within the scope of the invention as long as the encoded oligo or polypeptide demonstrates HIV antigenicity. The HIV polypeptide molecules encoded by the polynucleotide sequences according to the invention preferably represent a fragment of at least 50% of the length of the native protein, which fragment may contain mutations but which retains at least one HIV epitope and demonstrates HIV antigenicity. Similarly, immunogenic derivatives according to the invention must demonstrate HIV antigenicity. Preferred immunogenic derivatives

provide some potential advantage over the native protein such as reduction or removal of a function of the native protein which is undesirable in a vaccine antigen such as enzyme activity (RT), transactivating activity (Tat), or CD4 downregulation (Nef). The polynucleotide sequences are preferably codon optimised for mammalian cells, in
5 line with preferred aspects of the invention.

HIV envelope proteins such as gp120 expressed in a mammalian cell will normally be glycosylated. Advantageously, the polynucleotide according to the invention comprises a gp120 encoding sequence which is adapted to reduce or prevent
10 glycosylation in a mammalian target cell, particularly a human target cell. Glycosylation may be reduced or prevented in a number of different ways, for example by removal of or mutation of the glycosylation sites or by removing the native secretion signal. Preferably in the polynucleotide construct according to the invention the gp120 or other form of HIV envelope protein lacks a functional
15 secretion signal. The secretion signal may vary in length between HIV isolates, for example it is 30 amino acids long in the W61D isolate described herein, but may be more or less than that for different isolates. Generally the secretion signal is clearly delineated and will be removed in its entirety, although this is not necessarily the case. A sufficient amount of the signal will be removed to prevent its function of taking the
20 envelope protein to the cellular machinery responsible for glycosylation. This can be easily tested for.

Preferred polynucleotide sequences are selected from the group:

- 25 1. gp120 codon optimised, minus secretion signal
2. gp120 codon optimised, minus secretion signal – tr Nef
3. gp120 codon optimised, minus secretion signal – tr Nef – mTat
4. gp120 codon optimised, minus secretion signal – Nef - mTat
5. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef
- 30 6. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef - mTat
7. gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-mTat
8. gp120 codon optimised, minus secretion signal - p17/24 gag - mNef-mTat

9. gp120 codon optimised, minus secretion signal - p17/24 gag - L1Nef-mTat
10. gp120 codon optimised, minus secretion signal - p17/24 gag - L2Nef-mTat
11. gp120 codon optimised, minus secretion signal - p17/24 gag - LLNef-mTat
12. gp120 codon optimised, minus secretion signal - p17/24 gag - mLLNef-mTat
- 5 13. gp120 codon optimised, minus secretion signal - p17/24 gag - mL1Nef-mTat
14. gp120 codon optimised, minus secretion signal - p17/24 gag - mL2Nef-mTat
15. gp120 codon optimised - trNef
16. gp120 codon optimised - trNef-mTat
17. gp120 codon optimised - Nef-mTat
- 10 18. Nef-mTat- gp120 codon optimised
19. trNef-mTat- gp120 codon optimised
20. gp120 codon optimised - p17/24 Gag – tr Nef
21. gp120 codon optimised – p17/24 Gag – tr Nef-mTat

15 mNef = deletion of G2 to give non-myristoylated Nef

L1 Nef = L174A mutation in Nef

L2Nef = L175A mutation in Nef

LLNef = L174A and L175A mutations in Nef

TrNef = Nef devoid of nucleotides encoding terminal amino acids 1-65

20 mRT = Reverse Transcriptase mutated to remove biological activity (W229K). RT is codon optimised

Gag = Gag codon optimised.

The invention preferably relates to HIV-1. It is preferred that the constructs described
25 herein are derived from an HIV clade B or clade C, particularly clade B.

Preferably the promoter is the promoter from HCMV IE gene, more particularly wherein the 5' untranslated region of the HCMV IE gene comprising exon 1 is included as described in WO 02/36792.

30

In another aspect the invention provides a polynucleotide encoding an HIV Tat molecule or fragment or immunogenic derivative thereof in a fusion with at least two

further HIV antigens, preferably including gp120 and Nef or fragments or immunogenic derivatives thereof, and optionally including Gag and/or RT or fragments or immunogenic derivatives thereof. Also provided are Tat-containing fusions encoded by these polynucleotides.

5

In another aspect the invention provides a vector comprising the polynucleotide sequences described herein. The polynucleotide sequence is preferably DNA and is preferably contained within a vector which is a double stranded DNA plasmid . Alternative vectors are described hereinbelow and include in particular adenovirus vectors such as chimp derived adenovirus vectors Pan 9 or Pan 5, 6 and 7, preferably where these are replication defective such that they cannot replicate in the target cells.

In yet another aspect the invention provides a polypeptide encoded by a polynucleotide or vector as described herein.

15

In one embodiment the invention provides a fusion protein comprising an HIV envelope protein or a fragment or immunogenic derivative thereof and at least one additional HIV non-structural or capsid protein or fragment or immunogenic derivative thereof. Said additional HIV protein is preferably selected from Nef, Gag,

20 RT and Tat.

In another embodiment the invention provides a fusion protein as defined herein, expressed from a polynucleotide which is codon optimised for mammalian cells.

25 In a further aspect the invention provides pharmaceutical compositions comprising the nucleotide sequences and vectors and polypeptides described herein, together with a pharmaceutically acceptable excipient, diluent, carrier or adjuvant. In a preferred embodiment the polynucleotide, preferably in the form of a DNA vector and preferably comprising at least one codon optimised HIV sequence, is present in a
30 composition comprising a plurality of particles, preferably beads such as gold beads, onto which the DNA is coated.

Delivery of polynucleotides according to the invention is preferably carried out by particle mediated delivery, particularly via a bombardment approach.

It is envisaged that the vectors according to the invention may be utilised with

5 immunostimulatory agents, preferably but not necessarily administered at the same time as the vectors and preferably formulated together in the compositions according to the invention.

10 Immunostimulatory agents for use in the invention include, but this list is by no means exhaustive and does not preclude other agents: synthetic imidazoquinolines such as imiquimod [S-26308, R-837], (Harrison, et al. ‘Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine’, Vaccine 19: 1820-1826, (2001)); and resiquimod [S-28463, R-848] (Vasilakos, et al. ‘Adjuvant activites of immune response modifier R-848: Comparison with CpG ODN’, Cellular

15 immunology 204: 64-74 (2000).), Schiff bases of carbonyls and amines that are constitutively expressed on antigen presenting cell and T-cell surfaces, such as tucaresol (Rhodes, J. et al. ‘ Therapeutic potentiation of the immune system by costimulatory Schiff-base-forming drugs’, Nature 377: 71-75 (1995)), cytokine, chemokine and co-stimulatory molecules as either protein or peptide or DNA, this

20 would include pro-inflammatory cytokines such as GM-CSF, IL-1 alpha, IL-1 beta, TGF- alpha and TGF – beta, Th1 inducers such as interferon gamma, IL-2, IL-12, IL-15 and IL-18, Th2 inducers such as IL-4, IL-5, IL-6, IL-10 and IL-13 and other chemokine and co-stimulatory genes such as MCP-1, MIP-1 alpha, MIP-1 beta, RANTES, TCA-3, CD80, CD86 and CD40L, other immunostimulatory targeting

25 ligands such as CTLA-4 and L-selectin, apoptosis stimulating proteins and peptides such as Fas, (49), synthetic lipid based adjuvants, such as vaxfectin, (Reyes et al., ‘Vaxfectin enhances antigen specific antibody titres and maintains Th1 type immune responses to plasmid DNA immunization’, Vaccine 19: 3778-3786) squalene, alpha-tocopherol, polysorbate 80, DOPC and cholesterol, endotoxin, [LPS], Beutler, B.,

30 ‘Endotoxin, ‘Toll-like receptor 4, and the afferent limb of innate immunity’, Current Opinion in Microbiology 3: 23-30 (2000)) ; CpG oligo- and di-nucleotides, Sato, Y. et al., ‘Immunostimulatory DNA sequences necessary for effective intradermal gene

immunization', *Science* 273 (5273): 352-354 (1996). Hemmi, H. et al., 'A Toll-like receptor recognizes bacterial DNA', *Nature* 408: 740-745, (2000) and other potential ligands that trigger Toll receptors to produce appropriate Th1-inducing cytokines, such as synthetic Mycobacterial lipoproteins, Mycobacterial protein p19,
5 peptidoglycan, teichoic acid and lipid A.

A preferred immunostimulatory agent for use with the invention is GM-CSF. This may be employed in the form of a polynucleotide expressing GM-CSF which is co-administered with the DNA vaccine of the invention. A DNA plasmid encoding GM-
10 CSF may be present in a pharmaceutical composition comprising the polynucleotide(s) according to the invention.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a Lipid A derivative such as monophosphoryl lipid A, or preferably 3-de-
15 O-acylated monophosphoryl lipid A. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555,
20 WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins.
25

According to a further aspect of the invention, a host cell comprising a polynucleotide sequence according to the invention, or an expression vector according to the invention, is provided. The host cell may be for example bacterial e.g. *E. coli*, mammalian e.g. human, or may be an insect cell. Mammalian cells comprising a
30 vector according to the present invention may be cultured cells transfected *in vitro* or may be cells transfected *in vivo* by administration of the vector to the mammal.

By codon optimisation is meant that the DNA sequence is optimised to resemble the codon usage of genes in mammalian cells. In particular, the codon usage in the sequence is optimised to resemble that of highly expressed human genes.

5 The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter “codons” which represent the amino acids the proteins encoded in an organism’s genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons
10 representing “stop” signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different
15 species show a different bias in their codon selection and, furthermore, utilisation of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly
20 biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in *E. coli* or a foreign or recombinant gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons or an abundance of codons which are rarely observed
25 in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

In an embodiment of the present invention there is provided a gp120 polynucleotide sequence which encodes a substantially non-glycosylated gp120 amino acid sequence,
30 wherein the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian genes. Preferably the polynucleotide sequence is a DNA

sequence. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

In the polynucleotides of the present invention, the codon usage pattern is altered from
5 that typical of human immunodeficiency viruses to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura
10 et.al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus
15 each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the
20 total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

25

According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude rare codons. Rare codons can be defined as codons representing <20% or more preferably representing <10% of the codons used for a particular amino acid in highly expressed genes of the target organism. Alternatively rare codons may
30 be defined as codons with a relative synonymous codon usage (RSCU) value of <0.3 or more preferably <0.2 in highly expressed genes of the target organism. An RSCU value is the observed number of codons divided by the number expected if all codons

for that amino acid were used equally frequently. An appropriate definition of a rare codon would be apparent to a person skilled in the art.

A polynucleotide of the present invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. Preferably also the codon usage coefficient will be less than 1.0, preferably less than 0.9 and more preferably less than 0.8. Thus a codon usage coefficient between 0.5 and 0.9 or between 0.5 and 0.8 is most preferred. Codon usage tables for human can also be found in Genbank.

10

In comparison, a highly expressed beta actin gene has a codon usage coefficient of 0.747.

The codon usage table for a homo sapiens is set out below:

15

Codon usage for human (highly expressed) genes 1/24/91 (human_high.cod)

	AmAcid	Codon	Number	/1000	Fraction	..
20	Gly	GGG	905.00	18.76	0.24	
	Gly	GGA	525.00	10.88	0.14	
	Gly	GGT	441.00	9.14	0.12	
	Gly	GTC	1867.00	38.70	0.50	
25	Glu	GAG	2420.00	50.16	0.75	
	Glu	GAA	792.00	16.42	0.25	
	Asp	GAT	592.00	12.27	0.25	
	Asp	GAC	1821.00	37.75	0.75	
30	Val	GTG	1866.00	38.68	0.64	
	Val	GTA	134.00	2.78	0.05	
	Val	GTT	198.00	4.10	0.07	
	Val	GTC	728.00	15.09	0.25	
35	Ala	GCG	652.00	13.51	0.17	
	Ala	GCA	488.00	10.12	0.13	
	Ala	GCT	654.00	13.56	0.17	
	Ala	GCC	2057.00	42.64	0.53	

	Arg	AGG	512.00	10.61	0.18
	Arg	AGA	298.00	6.18	0.10
	Ser	AGT	354.00	7.34	0.10
	Ser	AGC	1171.00	24.27	0.34
5					
	Lys	AAG	2117.00	43.88	0.82
	Lys	AAA	471.00	9.76	0.18
	Asn	AAT	314.00	6.51	0.22
	Asn	AAC	1120.00	23.22	0.78
10					
	Met	ATG	1077.00	22.32	1.00
	Ile	ATA	88.00	1.82	0.05
	Ile	ATT	315.00	6.53	0.18
	Ile	ATC	1369.00	28.38	0.77
15					
	Thr	ACG	405.00	8.40	0.15
	Thr	ACA	373.00	7.73	0.14
	Thr	ACT	358.00	7.42	0.14
	Thr	ACC	1502.00	31.13	0.57
20					
	Trp	TGG	652.00	13.51	1.00
	End	TGA	109.00	2.26	0.55
	Cys	TGT	325.00	6.74	0.32
	Cys	TGC	706.00	14.63	0.68
25					
	End	TAG	42.00	0.87	0.21
	End	TAA	46.00	0.95	0.23
	Tyr	TAT	360.00	7.46	0.26
	Tyr	TAC	1042.00	21.60	0.74
30					
	Leu	TTG	313.00	6.49	0.06
	Leu	TTA	76.00	1.58	0.02
	Phe	TTT	336.00	6.96	0.20
	Phe	TTC	1377.00	28.54	0.80
35					
	Ser	TCG	325.00	6.74	0.09
	Ser	TCA	165.00	3.42	0.05
	Ser	TCT	450.00	9.33	0.13
	Ser	TCC	958.00	19.86	0.28
40					
	Arg	CGG	611.00	12.67	0.21
	Arg	CGA	183.00	3.79	0.06
	Arg	CGT	210.00	4.35	0.07
	Arg	CGC	1086.00	22.51	0.37
45					

Gln	CAG	2020.00	41.87	0.88
Gln	CAA	283.00	5.87	0.12
His	CAT	234.00	4.85	0.21
His	CAC	870.00	18.03	0.79
5				
Leu	CTG	2884.00	59.78	0.58
Leu	CTA	166.00	3.44	0.03
Leu	CTT	238.00	4.93	0.05
Leu	CTC	1276.00	26.45	0.26
10				
Pro	CCG	482.00	9.99	0.17
Pro	CCA	456.00	9.45	0.16
Pro	CCT	568.00	11.77	0.19
Pro	CCC	1410.00	29.23	0.48
15				

According to a further aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a polynucleotide sequence according to the first aspect of the invention, in particular wherein the codon usage pattern of the gp120 polynucleotide sequence is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector may be suitable for driving expression of heterologous DNA in bacterial insect or mammalian cells, particularly human cells. In one embodiment, the expression vector is p7313 (see Figure 1).

25 In a further aspect the invention provides a method of treating or preventing HIV infections, any symptoms or diseases associated therewith, comprising administering a safe and effective amount of a polynucleotide, a vector, a polypeptide or a pharmaceutical composition according to the invention.

30 Administration of the pharmaceutical composition may take the form of one or of more than one individual doses, for example as repeat doses of the same DNA plasmid, or in a heterologous “prime-boost” vaccination regime, particularly a therapeutic vaccination regime. A heterologous prime-boost regime uses 35 administration of different forms of vaccine in the prime and the boost, each of which may itself include two or more administrations. Preferably but not necessarily the

priming and boosting composition comprise the same antigens or different forms of the same antigens. The priming composition and the boosting composition will anyway have at least one antigen in common, although it is not necessarily an identical form of the antigen, it may be a different form of the same antigen. An 5 example of different forms of the same antigen is in the case of a polynucleotide encoding a gp120 which lacks a functional signal sequence and is substantially non-glycosylated in mammalian cells, and a poplypeptide which is gp120 with its signal sequence and which is glycosylated. A full length and a truncated version of the same protein, or a mutated and a non-mutated form of the same protein, may also be 10 considered different forms of the same antigen for the purposes of a prime-boost format according to the invention.

In one example of a prime-boost regime the “prime” vaccination may be via particle mediated DNA delivery of a priming composition which comprises a polynucleotide 15 according to the present invention, preferably incorporated into a plasmid vector, while the “boost” administration may be of a boosting composition comprising a recombinant viral vector comprising the same polynucleotide sequence or a polynucleotide encoding at least one of the same antigens encoded by the priming composition. Alternatively the boosting may be carried out with at least one of the 20 same antigens in the form of the protein in adjuvant. Conversely the priming may be with a priming composition comprising the viral vector or with a protein formulation typically a protein formulated in adjuvant, and the boost a DNA vaccine of the present invention.

25 A preferred prime-boost format for use with the polynucleotides according to the present invention is selected from:

- Protein prime / live vector boost
- Live vector prime / protein boost
- Protein prime / DNA plasmid boost

30 DNA plasmid prime / protein boost

- Live vector prime / DNA plasmid boost
- DNA plasmid prime / live vector boost

Preferred live vectors include live virus vectors in particular adenovirus vectors as described herein.

5 Preferably the priming and boosting compositions comprise, in addition to the antigen(s) a suitable adjuvant, which may be different according to the particular composition.

Both the priming composition and the boosting composition may be delivered in more
10 than one dose. Furthermore the initial priming and boosting doses may be followed up with further doses which may be alternated to result in e.g. a DNA plasmid prime / protein boost / further DNA plasmid dose / further protein dose.

The invention further provides a process for the production of a polynucleotide as
15 described herein comprising linking a nucleotide sequence encoding an HIV envelope protein such as gp120, in particular a substantially non-glycosylated gp120 or a fragment or immunogenic derivative thereof, and a sequence encoding an HIV non-structural protein such as Nef, RT or Tat or an HIV capsid protein such as Gag, or fragments or immunogenic derivatives thereof, to a heterologous promoter sequence.

20

As discussed above, the present invention includes expression vectors that comprise the nucleotide sequences of the invention. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements,
25 such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors and how to construct them would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook *et al.* Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press. (1989).

30

Preferably, a polynucleotide of the invention, or for use in the invention in a vector, is operably linked to a control sequence which is capable of providing for the expression

of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in
5 such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

A nucleic acid sequence of the present invention may be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are
10 discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and non-viral vector systems can be used. The vectors may be, for example, plasmids, artificial chromosomes (e.g. BAC, PAC, YAC), virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more
15 selectable marker genes, for example an ampicillin or kanamycin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell e.g. for the production of protein encoded by the vector. The vectors may also be adapted to be used *in vivo*, for
20 example in a method of DNA vaccination or of gene therapy.

Examples of suitable viral vectors include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral such as herpes simplex viral, alpha-viral, pox viral such as Canarypox and vaccinia-viral based systems. Gene transfer techniques using these
25 viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide of the invention into the host genome, although such recombination is not preferred. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. Vectors capable of driving expression in insect cells (for example baculovirus vectors), in
30 human cells, yeast or in bacteria may be employed in order to produce quantities of the HIV protein encoded by the polynucleotides of the present invention, for example for use as subunit vaccines or in immunoassays.

In a preferred embodiment the adenovirus used as a live vector is a replication defective simian adenovirus. Typically these viruses contain an E1 deletion and can be grown on cell lines that are transformed with an E1 gene. Preferred Simian
5 adenoviruses are viruses isolated from Chimpanzee. In particular C68 (also known as Pan 9) (See US patent No 6083 716) and Pan 5, 6 and Pan 7 (WO 03/046124) are preferred for use in the present invention. Thus these vectors can be manipulated to insert a heterologous gene of the invention such that the gene product maybe expressed. The use, formulation and manufacture of such recombinant adenoviral
10 vectors is set forth in detail in WO 03/046142.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which expression is designed. For example, mammalian promoters include the metallothionein promoter which can be induced in response to
15 heavy metals such as cadmium, and the β -actin promoter. Viral promoters such as the SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (IE) promoter, rous sarcoma virus LTR promoter, adenovirus promoter, or a HPV promoter, particularly the HPV upstream regulatory region (URR) may also be used.
All these promoters are well described and readily available in the art.

20 A preferred promoter element is the CMV immediate early promoter devoid of intron A, but including exon 1. Accordingly there is provided a vector comprising a polynucleotide of the invention under the control of HCMV IE early promoter. A suitable HCMV IE promoter is described in WO 02/36792.

25 Non-viral based systems include direct administration of nucleic acids, microsphere encapsulation technology, poly(lactide-co-glycolide) and liposome-based systems.

30 The polynucleotides according to the invention have utility in the production by expression of the encoded proteins, which expression may take place *in vitro*, *in vivo* or *ex vivo*. The nucleotides may therefore be involved in recombinant protein synthesis, for example to increase yields, or indeed may find use as therapeutic agents

in their own right, utilised in DNA vaccination techniques. Where the polynucleotides of the present invention are used in the production of the encoded proteins *in vitro* or *ex vivo*, cells, for example in cell culture, will be modified to include the polynucleotide to be expressed. Such cells include transient, or preferably stable
5 mammalian cell lines. Particular examples of cells which may be modified by insertion of vectors encoding for a polypeptide according to the invention include mammalian HEK293T, CHO, HeLa, 293 and COS cells. Preferably the cell line selected will be one which is stable. Expression may be achieved in transformed oocytes. A polypeptide may be expressed from a polynucleotide of the present
10 invention, in cells of a transgenic non-human animal, preferably a mouse. A transgenic non-human animal expressing a polypeptide from a polynucleotide of the invention is included within the scope of the invention.

The invention further provides a method of vaccinating a mammalian subject which
15 comprises administering thereto an effective amount of a vaccine or vaccine composition according to the invention. Preferably, expression vectors for use in DNA vaccines, vaccine compositions and immunotherapeutics will be plasmid vectors or live viral vectors.

20 DNA vaccines may be administered in the form of "naked DNA", for example in a liquid formulation administered using a syringe or high pressure jet, or DNA formulated with liposomes or an irritant transfection enhancer, or by particle mediated DNA delivery (PMDDor particle mediated imunotherapy delivery PMID) as described in more detail herein. All of these delivery systems are well known in the
25 art. The vector may be introduced to a mammal for example by means of a viral vector delivery system.

The compositions of the present invention can be delivered by a number of routes such as intramuscularly, subcutaneously, intraperitoneally, intravenously or mucosally.

30 The invention further provides an intradermal delivery device comprising a pharmaceutical composition described herein.

In a preferred embodiment, the composition is delivered intradermally. In particular, the composition is delivered by means of a gene gun particularly using particle bombardment administration techniques which involve coating the vector on to beads 5 (eg gold beads) which are then administered under high pressure into the epidermis. This is described, for example, in Haynes et al, J Biotechnology 44: 37-42 (1996).

Numerous methods of carrying out a particle bombardment approach are known, see for example WO 91/07487. In one illustrative example, gas-driven particle 10 acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, 15 such as polynucleotide, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest, typically the skin. The particles are preferably gold beads of a 0.4 – 4.0 µm, more preferably 0.6 – 2.0 µm diameter and the DNA conjugate coated onto these and then encased in a cartridge or cassette for placing into the delivery device.

20 In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 25 5,993,412.

The vectors which comprise the nucleotide sequences encoding antigenic peptides are administered in such amount as will be prophylactically or therapeutically effective. The quantity to be administered, is generally in the range of one picogram to 1 30 milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery, and 100 nanograms to 10 milligrams for other routes of nucleotide per dose. The exact quantity may vary considerably depending on the weight of the patient being

immunised and the route of administration.

It is possible for the immunogen component comprising the nucleotide sequence encoding the antigenic peptide, to be administered on a one off basis or to be

5 administered repeatedly, for example, between 1 and 7 times, preferably between 1 and 4 times, at intervals between about 1 day and about 18 months. Further administrations may also be given as necessary to maintain immune responses for the lifetime of the patient. However, this treatment regime will be significantly varied depending upon the size of the patient concerned, the amount of nucleotide sequence

10 administered, the route of administration, and other factors which would be apparent to a skilled medical practitioner. The patient may receive one or more other anti HIV retroviral drugs as part of their overall treatment regime. Additionally the nucleic acid immunogen may be administered with an adjuvant.

15 The adjuvant component specified herein can similarly be administered via a variety of different administration routes, such as for example, via the oral, nasal, pulmonary, intramuscular, subcutaneous, intradermal or topical routes. Preferably, the adjuvant component is administered via the intradermal or topical route, most preferably by a topical route. This administration may take place between about 14 days prior to and

20 about 14 days post administration of the nucleotide sequence, preferably between about 1 day prior to and about 3 days post administration of the nucleotide sequence.

The adjuvant component is, in one embodiment, administered substantially simultaneously with the administration of the nucleotide sequence. By "substantially simultaneous" what is meant is that administration of the adjuvant component is preferably at the same time as administration of the nucleotide sequence, or if not, it is at least within a few hours either side of nucleotide sequence administration. In the most preferred treatment protocol, the adjuvant component will be administered substantially simultaneously with administration of the nucleotide sequence.

25 Obviously, this protocol can be varied as necessary, in accordance with the type of variables referred to above. It is preferred that the adjuvant is a 1H – imidazo [4,5c] quinoline – 4 – amine derivative such as imiquimod. Typically imiquimod will be

presented as a topical cream formulation and will be administered according to the above protocol.

Once again, depending upon such variables, the dose of administration of the derivative will also vary, but may, for example, range between about 0.1 mg per kg to about 100 mg per kg, where “per kg” refers to the body weight of the mammal concerned. This administration of the 1H-imidazo[4,5-c]quinolin-4-amine derivative would preferably be repeated with each subsequent or booster administration of the nucleotide sequence. Most preferably, the administration dose will be between about 1 mg per kg to about 50 mg per kg. In the case of a “prime-boost” scheme as described herein, the imiquimod or other 1H-imidazo[4,5-c]quinolin-4-amine derivative may be administered with either the prime or the boost or with both the prime and the boost.

While it is possible for the adjuvant component to comprise only 1H-imidazo[4,5-c]quinolin-4-amine derivatives to be administered in the raw chemical state, it is preferable for administration to be in the form of a pharmaceutical formulation. That is, the adjuvant component will preferably comprise the 1H-imidazo[4,5-c]quinolin-4-amine combined with one or more pharmaceutically acceptable carriers, and optionally other therapeutic ingredients. The carrier(s) must be “acceptable” in the sense of being compatible with other ingredients within the formulation, and not deleterious to the recipient thereof. The nature of the formulations will naturally vary according to the intended administration route, and may be prepared by methods well known in the pharmaceutical art. All methods include the step of bringing into association a 1H-imidazo[4,5-c]quinolin-4-amine derivative with an appropriate carrier or carriers. In general, the formulations are prepared by uniformly and intimately bringing into association the derivative with liquid carriers or finely divided solid carriers, or both, and then, if necessary, shaping the product into the desired formulation. Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a pre-determined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-

water liquid emulsion or a water-in-oil emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more
5 accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

10

The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient.

Formulations for injection via, for example, the intramuscular, intraperitoneal,
15 intradermal, or subcutaneous administration routes include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or
20 multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. Formulations suitable for pulmonary
25 administration via the buccal or nasal cavity are presented such that particles containing the active ingredient, desirably having a diameter in the range of 0.5 to 7 microns, are delivered into the bronchial tree of the recipient. Possibilities for such formulations are that they are in the form of finely comminuted powders which may conveniently be presented either in a piercable capsule, suitably of, for example,
30 gelatine, for use in an inhalation device, or alternatively, as a self-propelling formulation comprising active ingredient, a suitable liquid propellant and optionally, other ingredients such as surfactant and/or a solid diluent. Self-propelling

formulations may also be employed wherein the active ingredient is dispensed in the form of droplets of a solution or suspension. Such self-propelling formulations are analogous to those known in the art and may be prepared by established procedures. They are suitably provided with either a manually-operable or automatically functioning valve having the desired spray characteristics; advantageously the valve is of a metered type delivering a fixed volume, for example, 50 to 100 µL, upon each operation thereof.

In a further possibility, the adjuvant component may be in the form of a solution for use in an atomiser or nebuliser whereby an accelerated airstream or ultrasonic agitation is employed to produce a fine droplet mist for inhalation.

Formulations suitable for intranasal administration generally include presentations similar to those described above for pulmonary administration, although it is preferred for such formulations to have a particle diameter in the range of about 10 to about 200 microns, to enable retention within the nasal cavity. This may be achieved by, as appropriate, use of a powder of a suitable particle size, or choice of an appropriate valve. Other suitable formulations include coarse powders having a particle diameter in the range of about 20 to about 500 microns, for administration by rapid inhalation through the nasal passage from a container held close up to the nose, and nasal drops comprising about 0.2 to 5% w/w of the active ingredient in aqueous or oily solutions. In one embodiment of the invention, it is possible for the vector which comprises the nucleotide sequence encoding the antigenic peptide to be administered within the same formulation as the 1H-imidazo[4,5-c]quinolin-4-amine derivative. Hence in this embodiment, the immunogenic and the adjuvant component are found within the same formulation.

In one embodiment the adjuvant component is prepared in a form suitable for biostatic administration, and is administered via that route substantially simultaneously with administration of the nucleotide sequence. For preparation of formulations suitable for use in this manner, it may be necessary for the 1H-imidazo[4,5-c]quinolin-4-amine

derivative to be lyophilised and adhered onto, for example, particles such as gold beads which are suited for biolistic administration.

In an alternative embodiment, the adjuvant component may be administered as a dry
5 powder, via high pressure gas propulsion.

Even if not formulated together, it may be appropriate for the adjuvant component to be administered at or about the same administration site as the nucleotide sequence.

10 Other details of pharmaceutical preparations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania (1985), the disclosure of which is included herein in its entirety, by way of reference.

Suitable techniques for introducing the naked polynucleotide or vector into a patient
15 also include topical application with an appropriate vehicle. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). DNA uptake may be further facilitated by use of
20 facilitating agents such as bupivacaine, either separately or included in the DNA formulation. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US 5,697,901.

25 Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents include cationic agents, for example calcium phosphate and DEAE-Dextran and lipofectants, for example lipofectam and transfectam. The dosage of the nucleic acid to be administered can be altered.

30

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are

discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and non-viral vector systems can be used and are described above. Viral and non-viral delivery systems may be combined where it is desirable to provide booster injections after an initial vaccination, for example an initial "prime" DNA vaccination using a

5 non-viral vector such as a plasmid followed by one or more "boost" vaccinations using a viral vector or non-viral based system. Similarly the invention contemplates prime boost systems with the polynucleotide of the invention, followed by boosting with protein in adjuvant or vice versa.

10 A nucleic acid sequence of the present invention may also be administered by means of transformed cells. Such cells include cells harvested from a subject. The naked polynucleotide or vector of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The polynucleotide of the invention may integrate into nucleic acid already present in a

15 cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

20 The pharmaceutical compositions of the present invention may include adjuvant compounds as detailed above, or other substances which may serve to increase the immune response induced by the protein which is encoded by the DNA. These may be encoded by the DNA, either separately from or as a fusion with the antigen, or may be included as non-DNA elements of the formulation. Examples of adjuvant-type

25 substances which may be included in the formulations of the present invention include ubiquitin, lysosomal associated membrane protein (LAMP), hepatitis B virus core antigen, FLT3-ligand (a cytokine important in the generation of professional antigen presenting cells, particularly dendritic cells) and other cytokines such as IFN- γ and GMCSF. Other preferred adjuvants include imiquimod and resiquimod and

30 tucarasol, imiquimod being particularly preferred.

In a particular embodiment of the invention there is provided the use of a nucleic acid molecule as herein described for the treatment or prophylaxis of HIV infection, administered with imiquimod. The imiquimod is preferably administered topically, whereas the nucleic acid molecule is preferably administered by means of particle mediated delivery.

Accordingly the present invention also provides a method of treating a subject suffering from or susceptible to HIV infection, comprising administering a nucleic acid molecule as herein described and imiquimod.

10

The present invention will now be described by reference to the following examples:

EXAMPLES

Example 1: Plasmid Construction

5 1.1 Construction of gp120 containing plasmid

Recombinant gp120 glycoprotein described in the following examples is a synthetic form of the gp120 envelope protein of HIV-1 isolate W61D.

10 Codon Optimised (pgp120c):

The gene sequence was based on the gp120 sequence from the HIV-1 isolate W61D. This has a Codon Usage Coefficient of 0.297. Optimisation was performed using SynGene 2d, resulting in a CUC of 0.749 (Ertl, PF., Thomsen, LL. Technical issues in construction of nucleic acid vaccines. (2003) Methods 31(3); 199-206. SynGene uses a mathematical method for codon optimisation based on the relative frequencies of use. Briefly, codons are assigned value ranges according to their frequencies, so that more frequent codons have wider ranges, and placed in ascending frequency order. The value ranges are expressed as ≥ 0.000 , $\geq 0.0??$, $\geq 0.???$ And so on. A random number is generated between 0 and 0.99999. This is then used to select a codon, which will be the codon allocated the range within which the random number falls. To exclude rare codons the value 0.1 is added to the random number, so that it falls in the range 0.1-1.09999.

25 The gp120 sequence was split into 40 overlapping oligonucleotides, PCR assembled and recovered using the end primers. The gene was cloned into vector p7313-ie (shown in Figure 1) as a NotI-BamHI fragment and sequenced. Restriction fragments from three initial clones were combined to generate a single correct clone. The amino acid sequence and codon optimised DNA sequence are given in Figure 2.

30

1.2 Generation of Nef/Tat containing plasmids

Nef/Tat (pNTm and ptrNTm)

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244 (Figure 3). The plasmid pRIT 15244 is identical to pRIT 14913 described below except that the His tail has been deleted.

5

General

The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs since this gene is among those that are most closely related to the consensus Nef.

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

15 The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985. This *tat* gene bears mutations in the active site region (Lys41→Ala) and in RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

20 The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

Construction of vector pRIT14597 (encoding Nef-His protein).

25

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02.

NcoI

30 PRIMER 01: 5'ATCGTCCATG.GGT.GGC.AAG.TGG.T 3' [SEQ ID NO: 1]

SpeI

PRIMER 02: 5' CGGCTACTAGTGCAGTTCTGAA 3' [SEQ ID NO:2]

5 The integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified
in such a way that expression of heterologous protein starts immediately after the
native ATG codon of the AOX1 gene and will produce recombinant protein with a tail
of one glycine and six histidines residues. This PHIL-D2-MOD vector was
constructed by cloning an oligonucleotide linker between the adjacent AsuII and
10 EcoRI sites of PHIL-D2 vector. In addition to the His tail, this linker carries NcoI,
SpeI and XbaI restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

The *nef* PCR fragment obtained and the integrative PHIL-D2-MOD vector were both
restricted by NcoI and SpeI, purified on agarose gel and ligated to create the
15 integrative plasmid pRIT14597.

Construction of vector pRIT14913 (encoding fusion Nef-Tat mutant-His).

20 To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the
pCMVLys41/KGE plasmid with primers 03 and 04.

SpeI

PRIMER 03: 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3' [SEQ ID NO: 3]

25

SpeI

PRIMER 04: 5' CGGCTACTAGTTTCCCTTCGGGCCT 3' [SEQ ID NO: 4]

30

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913.

5 **1.3 Generation of PMID vectors for gp120 and Nef/Tat:**

gp120: Codon-optimised gp120 was provided as described above.

Nef/Tat (pNTm and ptrNTm):

10

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244 described above. The Tat in this plasmid contains three mutations to inactivate the transactivation function. The fusion contains full length Nef which has an immune modulatory function (Collins and Baltimore (1999)) that may be abrogated by N-terminal truncation. Therefore constructs were generated for both full length Nef/mutant Tat(pNTm) and truncated Nef/mutant Tat(ptrNTm), in which the first 65 amino acids of Nef were removed. These sequences were PCR amplified from pRIT15244 using primers:

20

5'Nef GAATT CGCGGCCGCATGGGTGGCAAGTGGTCAAAAAG
5'trNef GAATT CGCGGCCGCATGGTGGGTTTCCAGTCACACC
3'Tat GAATT CGGATCCTTATT CCTCGGGCCTGTCGGG
[SEQ ID NOS: 5,6,7]

25

The genes were cloned into vector p7313-ie as NotI-BamHI fragments and sequenced. PNTm and ptrNTm and the Nef/Tat and truncated Nef/Tat sequences are shown in Figures 4 and 5.

Dual expression vectors: (pRIX1 and pRIX2)

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The Nef/Tat and trNef/Tat expression cassettes were excised as ClaI-XmnI restriction fragments, and ligated into the ClaI and blunted Sse8387 I sites of the vector

containing the codon optimised gp120 (pgp120c) to provide single plasmids for expression of both proteins (pRIX1 and pRIX2 respectively).

Composition of plasmid p7313-ie (Figure 1)

5

The plasmid was constructed by replacing the beta-lactamase gene containing Eam1105I - PstI fragment of pUC19 (available from Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks, HP7 9NA) with an EcoRI fragment of pUC4K (Amersham-Pharmacia) containing the Kanamycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus IE1 promoter /enhancer, Intron A, was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and inserted into the SalI site of pUC19 as a XhoI -SalI fragment, incorporating the bovine growth hormone polyadenylation signal. Deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector (WO00/23592 - Powderject Vaccines Inc.). HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA, 78, 2606-10, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with *Bgl* II, Klenow polymerase treatment, digestion with *Bst*X I, digestion with *Nco* I, treatment with mung bean nuclease to remove overhang and further digestion with *Bst*X I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids) and the X gene start codon. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and coding sequences of the S antigen were excised and replaced with an oligonucleotide linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

Hind---NotI-- EcoRV- -NdeI- -BamHI
 AGCTTGCGGCCGCTAGCGATATCGGTACCATATGTCGACGGATCC....
ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGCCTAGGCCGG

5 -NheI- -KpnI- -SalI- ΔNotI

[SEQ ID NO: 8]

This polylinker was further extended by insertion of an additional oligonucleotide linker between the KpnI and SalI sites:

10

AspI- -MunI- NaeI- NdeI-- BglII-
 GTACCGGTCAATTGGCGCCGGCGCGCCATATGACGTAGATCTG----
 ----GCCAGTTAACCGCGGCCGCGCGGTATACTGCAGTCTAGACAGCT
 --AgeI- -NarI-- AatII- SalI

15 [SEQ ID NO: 9]

The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from David Hodgeson (Warwick University) and amplified by PCR using primers to place EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted 20 into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the amplified cer was verified against the Genbank entry M11411.

The HBV 3'UTR sequence between the promoter and polyadenylation signal was removed by PCR amplification of the polyadenylation signal using primers:

sense: CCATGGATCCGATCTTTCCCTCTGCC [SEQ ID NO: 10]

25 antisense: GTTAGGGTGAAAAGCTTCCGAGTGAGAGACAC [SEQ ID NO: 11]

The resulting product was cut with BamHI and XmnI and used to replace the corresponding fragment containing both the polyadenylation signal and the 3'UTR.

The Intron A sequence was removed from the plasmid by PCR amplification of the CMV promoter/enhancer using primers:

30 sense: GCTAGCCTGCAGGCTGACCGCCAAACGAC [SEQ ID NO: 12]

antisense: GTTCTCCATCGCGGCCGACTCTGGCACGGGG [SEQ ID NO: 13]

The resulting product was cut with Sse8387 I and NotI, and inserted back into the Sse8387 I and NotI sites of the parental vector.

5 **Example 2: Modification of gp120 and Nef/Tat(mut) expression vectors**

gp120 constructs were modified to reduce secretion of the protein.

Generation of constructs:

10 gp120 without a secretion signal (dsgp120, pRix 12 – see Figure 2 and 6)

The gp120 gene was PCR amplified from pgp120c using the following primers:

5'120ds: 5'GAATTCGCGGCCATGGCCGAGCAGCTGTGGTCACC

15 [SEQ ID NO: 14]

L01:

5'GAATTGGATCCTCATCTCTGCACGACGCCGCTGGCCGGT
GGGGGCCACG [SEQ ID NO: 15]

20 Fragments were amplified using PWO DNA polymerase (Roche) and the cycle:

95°C (30s) 95°C(30s) 50°C(30s) 72°C(90s) 72°C(120s) 4°C(hold)

____ repeat x20 ____/

25

The products were cut with NotI and BamHI and cloned into p7313-ie to give pRix12 (Figure 6).

Results

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In 293T cells the vector pRIX12, which lacks the secretion signal, makes a good amount of a 60kDa non-glycosylated protein that is not secreted.

5 **Example 3: Construction of vectors for expression of gp120 and Nef/Tat(mut) from a single plasmid**

Vector construction:

The gp120 Nef/Tat(m) constructs were generated by PCR stitching the gp120 and
10 Nef/Tat(m) or trNef/Tat(m) orfs.

5' and 3' Gp120, 5' and 3' Nef/Tat(m) and 5'trNef/Tat were amplified from pRix1.
3'trNef/Tat(m) was amplified from pNTm. The following primers were used:

15 3'120: (antisense to): GCCAAGCGCCGCGTCGTGCAGAGA [SEQ ID NO: 16]

5'120/NT:

GCCAAGCGCCGCGTCGTGCAGAGAATGGGTGGCAAGTGGTCAAAAAAGT
[SEQ ID NO: 17]

3'NT (antisense to): GGGGAGCCGACAGGCCCGAAGGAA [SEQ ID NO: 18]

20

5'NT/120:

GGGGAGCCGACAGGCCCGAAGGAAATGAAGGTCAAGGAGACCAGAAAG
[SEQ ID NO: 19]

5'120/trN:

25 GCCAAGCGCCGCGTCGTGCAGAGAATGGTGGTTCCAGTCAC
[SEQ ID NO: 20]

5'trNef:

GAATTCGCGGCCATGGTGGGTTCCAGTCACACC [SEQ ID
NO: 21]

30 L01:

GAATTGGATCCTCATCTCTGCACGACGCCGCTGGCCGGGTG
GGGCCACG [SEQ ID NO: 22]

L02:

ACCACCTTGTACTTGTACAGCTCGCTCCGCCAGTTATCCCTCATGTC
GCCGCCGCCGGGC [SEQ ID NO: 23]

5

Fragments were amplified using PWO DNA polymerase (Roche) and the cycle:

95°C (60s) 95°C(30s) 55°C(30s) 72°C(120s) 72°C(120s) 4°C(hold)

10 \underline{\hspace{1cm}} repeat x20 \underline{\hspace{1cm}} /

Primer L1 was used as the 3' primer for 3'gp120. However there were problems using this primer when stitching Nef/Tat or trNef/T to the 5' end of gp120 so primer L2 was used instead.

15

The stitched gp120-N/Tm and gp120-trN/Tm fragments were cut with NotI and BamHI and cloned into similarly cut p7313-ie. Due to the use of primer L2 rather than L1 the N/Tm-gp120 and trN/Tm-gp120 fragments lacked a BamHI site, so these were cut with NotI and AccI, and cloned into similarly cut pgp120c. All inserts were fully 20 verified by sequencing. The plasmids were designated pRix6 (gp120c NefTat^m), pRix11 (gp120c trNefTat^m), pRix7 (NefTatm gp120c) and pRix8 (trNefTat^m gp120) (Figures 23-26).

25 **Example 4: Construction of vectors to investigate the effects of glycosylation and secretion, inclusion of Tat and inclusion of Gag (p17/24) and Nef and RT on gp120 and gp120 fusions**

Vectors were constructed as shown in Figures 33 and 34(schematic).

30

pRix 28 and pRix29 (Figures 7 and 8)

pRix28 and 29 containing ds gp120c NefTat^m and ds gp120c trNefTat were generated by transferring the AccI-BamHI fragments from pRix6 (2315bp) and pRix11 (2123bp) into similarly cut pRix12 (ds gp120c).

5 pRix30 and pRix31 (Figure 27 and Figure 9)

To generate glycosylated and non-glycosylated fusion vectors of gp120c Nef without Tat, the NotI-KpnI fragment was transferred from pRix11 (1580bp) or pRix29 (1496bp) into similarly cut pRix15, a vector containing Tat/trNef.

10

(pRix15) - Tat(mut)trNef

The genes for Tat and trNef were PCR amplified from pNTm using the following primers:

15

5'Tat: 5'GAATT CGCGGCCGCATGGAGCCAGTAGATCCTAGAC [SEQ ID NO: 24]

3'Tat: 5'TTCCTTCGGGCCTGTCGGC [SEQ ID NO: 25]

5'trTN: GCCGACAGGCCCGAAGGAAATGGTGGGTTTCCAGTCACAC [SEQ ID NO: 26]

20 3'Nef: GAATT CGGATCCTTAGCAGTTCTGAAGTACTCCGG [SEQ ID NO: 27]

The individual genes were gel purified and then PCR stitched to give TmtrN using the end primers. The fusion was then digested with NotI and BamHI and cloned into p7313-ie.

25

pRix32 (Figure 28)

To generate the fusion containing p17/24, gp120 was PCR amplified from pgp120c using primers U1 and 3'120, p17/24-Nef was amplified from p73i-GN2 using primers 30 5'120G and 3'Nef, and the two were PCR stitched using U1 and 3'Nef. p73I-GN2 contained a synthetic codon optimised sequence of p17/p24 based on the sequence of HXB2 (GenBank entry K03455) and designed using SynGene and assembled from

overlapping oligonucleotides as described for codon optimised gp120 above, fused to HXB2 Nef, which had been obtained from plasmid pHXB Δ Pr (B.Maschera, E Furfine and E.D. Blair 1995 J.Virol 69 5431-5436) by PCR. Since the HXB2 nef gene in this plasmid contains a premature termination codon two overlapping PCRs were used to
5 repair the codon (TGA [stop] to TGG [Trp]). The position of the repaired codon is underlined in the sequence. The p17/p24/Nef gene was inserted into the NotI and BamHI sites of plasmid p7313ie. The coding sequence and map is given in Figure 10. A * marks the p24/trNef junction.

10 Primers:

U1:

GAATT CGCGGCCGCAATGAAGGTCAAGGAGACCAGAAAGAACTACCAGC
ATCTGTG [SEQ ID NO: 28]

3'120: TCTCTGCACGACGCCGCGCTTGGC [SEQ ID NO: 29] 5'120G:

15 GCCAAGCGCCGCGTCGTGGAGAGAATGGGTGCCGAGCTTCGGTAC [SEQ
ID NO: 30]

3'Nef: GAATT CGGATCCTTAGCAGTTCTGAAGTACTCCGG [SEQ ID NO: 31]

Initial cycle:

20 94°C(30s) 20x[94°C (30s) 50°C (30s) 68°C (180s)] 68°C (120s) 4°C (0s)

Using pfx polymerase with 1x enhancer.

Stitch:

94°C(30s) 20x[94°C (30s) 50°C (30s) 72°C (180s)] 72°C (120s) 4°C (0s)

25 Using Vent polymerase in standard conditions + 2mM MgCl₂.

The product was cut with NotI and BamHI and cloned into p7313-ie.

On sequencing the construct was found to have a error in the signal peptide, which
30 was corrected by transferring the 2560bp BstEII - KpnI fragment containing the back
of gp120 to the front of Nef into pRix30.

pRix 33 (Figure 11), pRix34 (Figure 29), and pRix35 (Figure 12)

The 2560bp BstEII - KpnI fragment containing the back of gp120 to the front of Nef was transferred to pRix31, pRix11 and pRix29 to make vectors pRix 33 (Figure 11),
5 34 and 35 (Figure 12) respectively.

pRix39 (gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-Tat –
Figure 13) and pRix40-47 (Constructs pRix 40-47 contain non-glycosylated gp120,
10 gag-p17/24, Nef and Tat(m) fusions with mutations in the miristoylation site and/or
dileucine motif of Nef)

A fragment containing gag p17/24 was PCR amplified from vector pRix35 using primers:

15 5'120G:

GCCAAGCGCCGCGTCGTGGAGAGAATGGGTGCCGAGCTTCGGTAC [SEQ
ID NO: 32]

and

p24AS: CAACACTCTGGCTTGTTGTCC [SEQ ID NO: 33]

20

Full length Nef was PCR amplified from pNTm using primers:

5'p24-N: GGACACAAAGCCAGAGTGTGATGGCAAGTGGTCAAAAAGTAG
[SEQ ID NO: 34]

and

25 3'Nef: GAATTGGATCCTTAGCAGTTCTGAAGTACTCCGG [SEQ ID NO: 35]

The two fragments were PCR stitched together using the end primers (5'120G and 3'Nef). The product was cut with SalI and KpnI, and the 423bp fragment containing part of p24 and Nef was used to replace the corresponding fragment in pRix35 to
30 make pRix39 (Figure 13).

pRix40 (Figure 14) was similarly constructed except primer 5'p24-N was replaced with primer:

5'p24-Ndm:

5 GGACACAAAGCCAGAGTGGATGGCAAGTGGTCAAAAAGTAG [SEQ ID NO: 36]
 G H K A R V L | M G K W S K S

This primer deletes the one G to destroy the miristoylation site at the start of Nef.

10 pRix41-44, 46 and 47 (Figures 15 to 20)

Mutations to the dileucine motif in Nef (L174L175) were made by PCR:

To insert the mutations, the portion of Nef 5' to the LL motif was PCR amplified
 15 using the 5'Nef primer and asNefLL

5 'Nef GAATTCGCGGCCGCATGGGTGGCAAGTGGTCAAAAAG [SEQ ID
 NO: 37]
 asNefLL (Antisense to)
 20 GCCAATAAAGGAGAGAACACCAGC [SEQ ID NO: 38]
 A N K G E N T S

Mutations to L174, L175 or both 174 and 175 were generated using forward primers

25 sNefL1 (L174A)
 GCCAATAAAGGAGAGAACACCAGCGCCTTACACCCTGTGAGCCTGCATG
 A N K G E N T S | A L H P V S L H
 [SEQ ID NO: 39]

30 sNefL2 (L175A)
 GCCAATAAAGGAGAGAACACCAGCTGGCACACCCTGTGAGCCTGCATG
 A N K G E N T S | L a H P V S L H
 [SEQ ID NO: 40]

SnefLL (LL174/5AA)
GCCAATAAAGGAGAGAACACCAGCGCCGCACACCTGTGAGCCTGCATG
A N K G E N T S| A A H P V S L H
5 [SEQ ID NO: 41]

and the 3'NT primer:

3'NT (antisense to):GGGGAGCCGACAGGCCGAAGGAA [SEQ ID NO: 42]

10 to amplify the 3' portion of Nef. The 5' and each of the 3' products were PCR stitched using the 5'Nef and 3'NT primers. These were cut with KpnI and SpeI and inserted into similarly cut pRix39 to generate pRix41 (L174A), pRix42 (L175A), and pRix43 (LL174/175A) in the absence of the myristoylation site mutation, or into pRix40 to
15 generate pRix44 (mLL174/175AA) pRix46 (mL174A) and pRix47 (mL175A) with the myristoylation site mutation.

pRix58 (Figure 21)

20 The gp120 fragment without signal sequence was PCR amplified from pgp120c using the primers:

5' ds120: GAATT CGCGGCCGC ATGGCCGAGCAGCTGTGGTCACC [SEQ ID NO: 43]

25 3'120: (antisense to): GCCAAGCGCCGCGTGCAGAGA [SEQ ID NO: 44]

the 5' end of RT (codon optimised and containing the W229K inactivating mutation) was PCR amplified from pt-rng (Figure 32 – see also WO 03/025003) using a 5' primer to insert a sequence homologous to 3'120, and a primer within RT

30 120RTf: GCCAAGCGCCGCGTGCAGAGAATGGGCCCATCAGTCCCATC [SEQ ID NO: 45]

RT3SR1: CGTCACGATGTTCACCTCCAGGCC [SEQ ID NO: 46]

The two products were PCR stitched using the end primers, and cut with NotI and NheI. The fragment was gel purified and used to replace the NotI -NheI fragment from
5 pT-rng.

pRix59 (Figure 22): the 3'Gag fragment was PCR amplified from pT-rng using a primer 5' to the MunI site in p24 and a 3' primer encoding the start of dsgp120, covering the position of the BstEII site near the 5' end:

10

GagMunf

GTGGCCCGAGAGCTGCATCCG [SEQ ID NO: 47]

GAG120R (Antisense to:)

15

Gag-----ds120

GGACACAAAGCCAGAGTGGATGGCCGAGCAGCTGTGGGTACCGTC

[SEQ ID NO: 48]

20

The product was cut with MunI and BstEII, and inserted into the 7113bp fragment from MunI-BstEII cut pRix54.

pRix48 and 49 (Figures 30 and 31)

25

The plasmids pRix 48 and 49 are equivalent to pRix39 and 41 except that they contain glycosylated gp120. To generate pRix48 and pRix49, the NotI – SallI fragments of pRix39 and pRix41 were replaced with the equivalent fragment from pRix32.

Results

30

Expression data is shown in Figures 33 and 35.

293T cell monolayers in 24 well plates were transfected with 1 μ g of each DNA indicated using Lipofectamine 2000 following the manufacturer's supplied protocol. After 24 hours the cells were detached and separated from the culture medium by centrifugation. Samples equivalent to 1x10⁴ cells or 12 μ l of medium were examined
5 by PAGE and Western blot.

The gp120c construct gave a highly glycosylated well secreted protein. Addition of c-terminal Nef/Tat fusions (pRix 6 and pRix11) resulted in a reduction of the intracellular protein levels and loss of secretion. Removal of the secretion signal from
10 gp120c (pRix12) gave a non-glycosylated non-secreted form of the protein.

As expected, fusion constructs with no secretion signal pRix28, 29, 31, 33 and 35, made non-glycosylated intracellular proteins in similar amounts, though expression from pRix35 was somewhat reduced. Surprisingly, when the secretion signal was
15 present in constructs pRix30, 32 and 34, only pRix34 failed to be secreted. It appears that the presence of Tat in the fusion inhibits secretion of the protein. The initial pRix32.1 construct had a point mutation resulting in poor expression. This was corrected in pRix32.7, which showed greatly improved expression.

20 For pRix40-47 the western blot in Figure 35 shows the expression of the dsgp120/Gag/Nef/Tat fusions with mutations in Nef in 293T cells 24hours post transfection with the plasmids indicated. Total cell extracts equivalent to ~1x10⁴ cells were loaded onto the gel. The blot was probed with an anti-nef antiserum.

25 For pRix48-49 the western blot in Figure 35 shows the expression of the gp120/Gag/Nef/Tat fusions with glycosylated gp120 in 293T cells 24hours post transfection with the plasmids shown. Total cell extracts equivalent to ~1x10⁴ cells were loaded onto the gel. The blot was probed with an anti-nef antiserum.

30 Similarly, expression data (anti-Nef) for the quadrivalent fusion proteins containing RT, Nef, Gag and dsgp120, compared to expression of the RT,Nef,Gag fusion alone, is shown in Figure 35.

Refs:

Andre S. Seed B. Eberle J. Schraut W. Bultmann A. Haas J. (1998) Increased
5 immune response elicited by DNA vaccination with a synthetic gp120 sequence with
optimized codon usage. *Journal of Virology.* 72(2):1497-503.

Vinner L. Nielsen HV. Bryder K. Corbet S. Nielsen C. Fomsgaard A. (1999) Gene
gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene
10 using mammalian codons. *Vaccine.* 17(17):2166-75

Collins KL. Baltimore D.(1999) HIV's evasion of the cellular immune response.
Immunological Reviews. 168:65-74.

15

Example 5: Preparation of plasmid-coated 'gold slurry' for 'gene gun' DNA cartridges

Plasmid DNA (approximately 1 μ g/ μ l), eg. 100 ug, and 2 μ m gold particles, eg. 50 mg,
20 (PowderJect), were suspended in 0.05M spermidine, eg. 100 ul, (Sigma). The DNA
was precipitated on to the gold particles by addition of 1M CaCl₂, eg. 100ul
(American Pharmaceutical Partners, Inc., USA). The DNA/gold complex was
incubated for 10 minutes at room temperature, washed 3 times in absolute ethanol, eg.
3 x 1 ml, (previously dried on molecular sieve 3A (BDH)). Samples were resuspended
25 in absolute ethanol containing 0.05mg/ml of polyvinylpyrrolidone (PVP, Sigma), and
split into three equal aliquots in 1.5 ml microfuge tubes, (Eppendorf). The aliquots
were for analysis of (a) 'gold slurry', (b) eluate- plasmid eluted from (a) and (c) for
preparation of gold/ plasmid coated Tefzel cartridges for the 'gene gun', (see Example
3 below). For preparation of samples (a) and (b), the tubes containing plasmid DNA /
30 'gold slurry' in ethanol / PVP were spun for 2 minutes at top speed in an Eppendorf
5418 microfuge, the supernatant was removed and the 'gold slurry' dried for 10
minutes at room temperature. Sample (a) was resuspended to 0.5 – 1.0 ug / ul of

plasmid DNA in TE pH 8.0, assuming approx. 50 % coating. For elution, sample (b) was resuspended to 0.5 – 1.0 ug / ul of plasmid DNA in TE pH 8.0 and incubated at 37°C for 30 minutes, shaking vigorously, and then spun for 2 minutes at top speed in an Eppendorf 5418 microfuge and the supernatant, eluate, was removed and stored at -5 20°C. The exact DNA concentration eluted was determined by spectrophotometric quantitation using a Genequant II (Pharmacia Biotech).

Example 6: Preparation of Cartridges for DNA immunisation

10 Preparation of cartridges for the Accell gene transfer device was as previously described (Eisenbraun et al DNA and Cell Biology, 1993 Vol 12 No 9 pp 791-797; Pertner et al). Briefly, plasmid DNA was coated onto 2 µm gold particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 15 4°C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with a total of 0.5 µg DNA/cartridge.

Example 7: PMID immunisations including using gp120-Nef-Tat triple fusion lacking gp120 secretion signal

Protocol: For PMID immunisations (DNA) cartridges were prepared for using standard methods as described in Examples 5 and 6. A DNA loading rate of 2, which will give approximately 0.5 µg DNA/cartridge was used and each immunisation 25 consisted of two shots. Balb/c mice were given a primary immunisation of DNA (using PMID). The mice were boosted 28 days later with DNA (using PMID). Mice were culled 7 days later and serum and spleens were collected. The splenocytes were harvested by teasing out the spleen cells and erythrocytes were lysed. The splenocytes were washed and counted. Specialised ELIspot plates (coated with interferon-gamma 30 capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37°C/5% CO₂ in the presence of a gp120 peptide, RT peptide or Gag peptide. The splenocytes were lysed and the plate developed using

standard procedures to demonstrate the number of interferon-gamma secreting cells present. Serum was analysed by ELISA assay to detect for specific antibodies. Results are shown in Figures 36-38.

5 Conclusion

Unexpectedly, the cellular immune response of mice immunised with dsgp120 (gp120 lacking secretion signal) expressing constructs was approximately double that of mice immunised with gp120 constructs (see Figure 36 and 37). This was consistent with the observation that in *in vitro* transfection studies the expression of dsgp120 had 10 remained largely cell associated, whereas gp120 had been excreted.

Inclusion of Tat (mutated Tat) in the dsgp120 constructs increased the cellular immune response to twice that of the dsgp120 constructs without Tat (Figures 36 and 37). Tat on its own did not affect the immune response to gp120, but acted 15 synergistically with dsgp120 to optimise the cellular response.

The inclusion of other HIV antigens in the constructs produced a balanced cellular response to all the different antigens included and thus broadened the immune response compared to the gp120 only vectors (Figure 38).

CLAIMS

1. A polynucleotide which comprises a sequence encoding an HIV envelope protein or fragment or immunogenic derivative thereof, fused to at least one sequence 5 encoding an HIV non-structural or capsid protein or fragment or immunogenic derivative thereof, operably linked to a heterologous promoter.
2. The polynucleotide according to claim 1 wherein the HIV envelope protein is gp120 or a fragment or immunogenic derivative thereof.
- 10 3. The polynucleotide according to claim 1 or claim 2 wherein the at least one non-structural or capsid protein or fragment or immunogenic derivative thereof is selected from one or more of Nef, Gag, RT or Tat.
- 15 4. The polynucleotide according to claim 3 wherein the gp120 encoding sequence is linked to a sequence encoding HIV RT or a fragment or immunogenic derivative thereof and a sequence encoding HIV Gag or fragment or immunogenic derivative thereof and a sequence encoding HIV Nef or a fragment or immunogenic derivative thereof to encode a gp120, RT, Gag and Nef-containing fusion protein.
- 20 5. The polynucleotide according to claim 4 wherein the fusion is selected from gp120-RT-Nef-Gag and RT-Nef-Gag-gp120.
- 25 6. The polynucleotide according to claim 3 wherein the gp120 encoding sequence is linked to a sequence encoding HIV Nef or an immunogenic derivative thereof to encode a gp120 and Nef-containing fusion protein.
- 30 7. The polynucleotide according to claim 6 wherein the gp120 sequence is further linked to a sequence encoding HIV Tat or a fragment or immunogenic derivative thereof to encode a gp120, Tat and Nef-containing fusion protein.
8. The polynucleotide according to claim 7 encoding a gp120-Nef-Tat fusion.

9. The polynucleotide according to claim 7 further comprising a sequence encoding HIV Gag or a fragment or immunogenic derivative thereof to encode a gp120-Gag-Nef-Tat fusion.

5

10. The polynucleotide according to any one of claims 3, 4, 5 or 9 wherein the Gag comprises p17 and/or 24.

11. The polynucleotide according to any one of claims 1 to 10 wherein the HIV envelope molecule is substantially non-glycosylated when expressed in a mammalian target cell.

12. The polynucleotide according to claim 11 wherein the HIV envelope molecule lacks a functional secretion signal.

15

13. The polynucleotide according to any one of claims 1 to 12 wherein one or more of the sequences encoding gp120, Nef, Gag, RT or Tat is or are codon optimised to resemble the codon usage in a highly expressed human gene.

20 14. A polynucleotide sequence selected from the group:

1. gp120 codon optimised, minus secretion signal – tr Nef
2. gp120 codon optimised, minus secretion signal – tr Nef – mTat
3. gp120 codon optimised, minus secretion signal – Nef - mTat
4. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef

25 7. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef - mTat

8. gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-mTat

9. gp120 codon optimised, minus secretion signal - p17/24 gag - mNef-mTat

10. gp120 codon optimised, minus secretion signal - p17/24 gag - L1Nef-mTat

11. gp120 codon optimised, minus secretion signal - p17/24 gag - L2Nef-mTat

30 12. gp120 codon optimised, minus secretion signal - p17/24 gag - LLNef-mTat

13. gp120 codon optimised, minus secretion signal - p17/24 gag - mLLNef-mTat

14. gp120 codon optimised, minus secretion signal - p17/24 gag - mL1Nef-mTat

15. gp120 codon optimised, minus secretion signal - p17/24 gag - mL2Nef-mTat
16. gp120 codon optimised - trNef
17. gp120 codon optimised - trNef-mTat
18. gp120 codon optimised - Nef-mTat
- 5 19. Nef-mTat- gp120 codon optimised
20. trNef-mTat- gp120 codon optimised
21. gp120 codon optimised - p17/24 Gag – tr Nef
22. gp120 codon optimised – p17/24 Gag – tr Nef-mTat
23. gp120 codon optimised, minus secretion signal – mRT- trNef – p17/24 Gag
- 10 24. mRT – trNef – p17/24 Gag – gp120 codon optimised, minum secretion signal

wherein RT and Gag are codon optimised.

15. The polynucleotide according to any one of claims 1 to 14 wherein the promoter is the promoter from HCMV IE gene.
16. The polynucleotide according to claim 15 wherein the 5' untranslated region between the promoter and coding polynucleotide comprises exon 1.
- 20 17. A vector comprising a polynucleotide as claimed in any one of claims 1 to 16.
18. The vector according to claim 17 which is a double-stranded DNA plasmid.
19. The vector according to claim 17 which is a replication defective adenovirus vector.
- 25 20. The vector according to claim 19 which is derived from Pan 9, 5, 6 or 7.
21. A fusion protein comprising an HIV envelope protein or fragment or immunogenic derivative thereof and at least one additional HIV protein or fragment or immunogenic derivative selected from non-structural or capsid proteins.
- 30

22. A fusion protein according to claim 21 wherein the fusion is selected from gp120-RT-Nef-Gag and RT-Nef-Gag-gp120.

23. A polypeptide encoded by the polynucleotide or vector according to any of 5 claims 1 to 20.

24. A pharmaceutical composition comprising a nucleotide sequence according to any one of claims 1 to 16, a vector of any one of claims 17 to 20, a fusion protein of claim 21 or 22 or a polypeptide of claim 23, and a pharmaceutically acceptable 10 excipient, diluent, carrier or adjuvant.

25. The pharmaceutical composition according to claim 24 wherein the carrier is a plurality of particles such as gold beads.

15 26. The pharmaceutical composition according to claim 24 or 25 for delivery in a prime boost format.

27. An intradermal delivery device comprising a pharmaceutical composition according to any one of claims 24 to 26.

20 28. A method of treating a patient suffering from or susceptible to a disease comprising administering a safe and effective amount of a pharmaceutical composition according to any one of claims 24 to 26.

25 29. A polynucleotide or a vector or fusion protein or polypeptide according to any one of claims 1 to 23 for use in medicine.

30. Use of a polynucleotide or a vector or fusion protein or polypeptide according to any one of claims 1 to 23 in the manufacture of a medicament for the treatment of 30 disease.

31. A process for the production of a polynucleotide according to any one of claims 1 to 16 comprising linking a nucleotide sequence encoding an HIV envelope protein or fragment or immunogenic derivative, preferably a non-glycosylated gp120 sequence, and a sequence encoding an HIV non-structural or capsid protein or fragment or immunogenic derivative, to a heterologous promoter sequence.
5
32. A polynucleotide encoding an HIV Tat molecule or fragment or immunogenic derivative in a fusion with at least two further HIV antigens.
- 10 33. The polynucleotide according to claim 32 wherein the two further HIV antigens include gp120 and Nef and optionally Gag and/or RT, or fragments or immunogenic derivatives thereof.
- 15 34. A Tat-containing fusion encoded by a polynucleotide according to claim 32 or
33.

Fig.1.

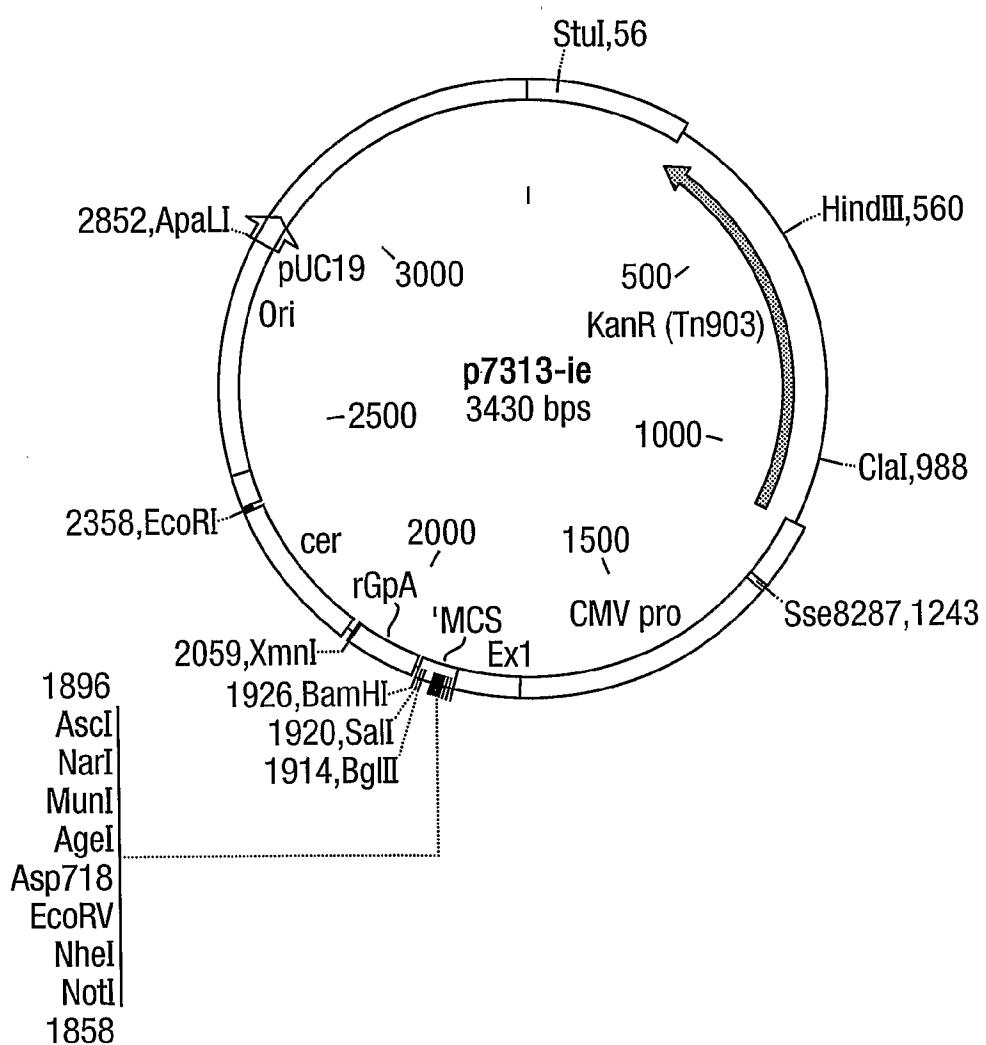
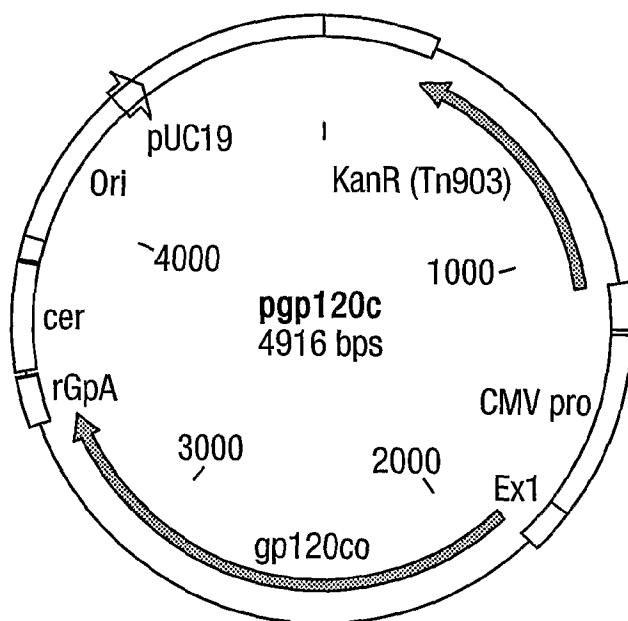


Fig.2.
Map of pgp 120c:



The amino acid sequence of the W61D gp120 is below. The signal sequence is underlined and bold, up to the predicted cleavage site between amino acids 29 and 30. This is the sequence removed in dsgp120 (pRix12 etc).

MKVKE TRKNY QHLW RWGTMLLGMLMICSAAEQLWVTVYYGPVWKEATTLFCASDAKAYDTEVHNWATH
 ACVPTDPNPQEVLGNVTEYFNMWKNMVDQMHEIDIISLWDQSLKPCVKLTPLCVTLDCDDVNTNSTTT
 SNGWTGEIRKGEIKNCSFNITTSIRDVKQKEYALFYNDVVPIDDDNATTKNKTRNFRLIH CNSS VMTQA
 CPKVS FEP IPIHYCAPAGFAILKCNNKTFDGKG LCTNVSTVQCTH GIPRVVSTQLLLNGSLAEEEVVIRSD
 NFMDNTKTIIVQLNESVAINTRPNNNTRKG IHI GPGR AFY AARKIIGDIRQAHCNLSRAQWNNTLKQIVI
 KLREHFGNKT IKFNQSSGGDPEIVRH SFNC GGEF YCDTTQ LFNSTW NGTEGN NTEGN STI L PCRI KQII
 NMWQEVGKAMYAPP IGGQIRCSSNITGLL TRDG GTEG NGTEN ETEI FRPGGGDM RDNWRSELYKYKVVK
 EPLGVAPTRAKRRVVQR [SEQ ID NO: 49]

The codon optimised DNA sequence for the W61D gp120 gene is:

ATGAAGGTCAAGGAGACCAGAAAGAACTACCA CAGCATCTGTGGCGCTGGGGCACCATGCTCCTGGGAATGCT
 GATGATCTGCTCCGCCCGAGCAGCTGTGGGT CACCGTCTACTACGGCGTGCTGTGGGAAGGAGGCCA
 CGACCAC CCTCTTCTGC CGAGCGACGCCAACGCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCAACATGTG
 GCTTGC GTGCCTACGGACCCCAACCCCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCAACATGTG
 GAAGAATAACATGGTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAGTCCCTGAAGCCCTGCG
 TGAAGCTGACGCCTCTCGCGTACACTGGACTGTGACGACGTCAACACCAACAGCACTACCACC
 AGCAACGGCTGGACCGGAGAGATTCGGAAGGGCGAGATCAAGAACTGCTCCTCAATATCACGACCTCGAT
 CAGAGACAAGGTG CAGAAGGAATACGGCCTGTTTATAATCTCGATGTGGTCCCCTCGACGACGACAATG
 CCACCAAGAACAGACGACGCGTAATTCAGACTCATTCACTGCAACAGCAGCGTATGACGCGAGGCC
 TGCCCCAAGGTGTCCCTCGAACCAATCCCGATCCATTACTGTGCCCCCTGCCGGATTCGCGATCCTCAAGTG
 TAACAAACAAGACCTCGACGGGAAGGGCCTGTGACCAACGTCAGCACGGTGCAGTGCACCCATGGCATCC

Fig.2 (Cont).

GCCCGTCGTGAGCACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTATCCGGTGGAC
 AACTTCATGGACAACACCAAGACAATCATCGTCAGCTGAACGAGTCTGTGGCGATTAACGTACCCGGCC
 TAACAACAACACCCGTAAAGGCATCCACATCGGGCCTGGACGGGCCTCTATGCCGCCGCAAGATCATCG
 GCGACATCCGGCAGGCCATTGCAACCTCTCCCGGCCAGTGGAAATAACACCCCTGAAGCAGATCGTGTAC
 AAGCTGAGAGAGCACTTGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCGAGATCGT
 GCGGCACTCCTCAACTGCGGGGGCGAGTTCTACTGCGATAACGACAGCTCTCAACTCCACCTGGA
 ACGGCACCGAGGGCAACAACACAGAGGGAAACTCCACTATCACCCCTCCCTGCCGCATCAAGCAGATCATC
 AACATGTGGCAGGAGGTGGAAAGGCCATGTATGCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCAA
 CATCACCGGCCTGCTGCTCACCAAGAGACGGGGGCCACGAGGGCAACGGCACGGAGAACGAGACGGAGATCT
 TCAGGCCCGGCGGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTG
 GAGCCGCTCGCGTGGCCCCACCCGGGCAAGCGCCGCGTGCAGAGATGA [SEQ ID NO: 50]

Fig.3.

Map of pRix15244:

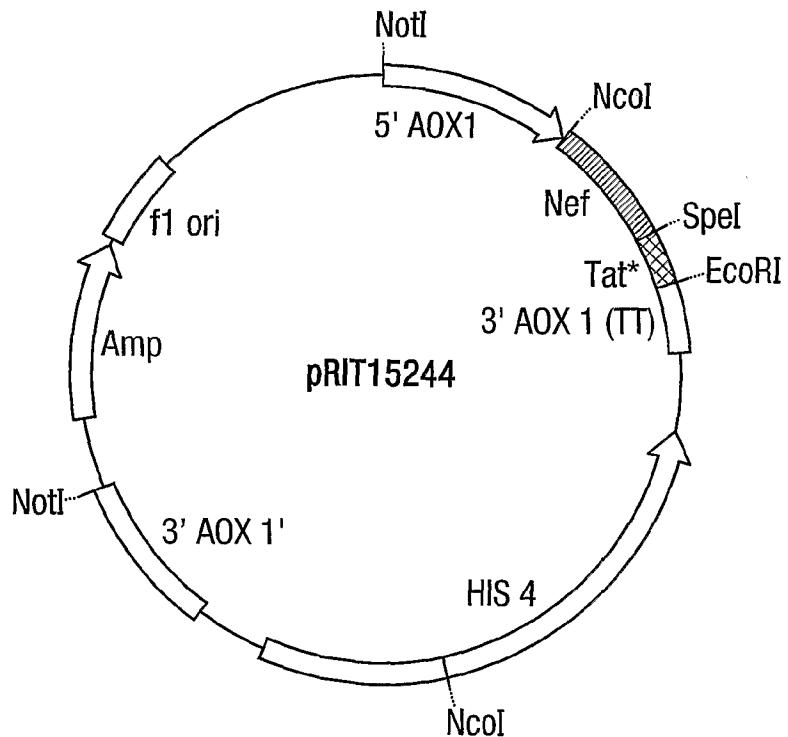
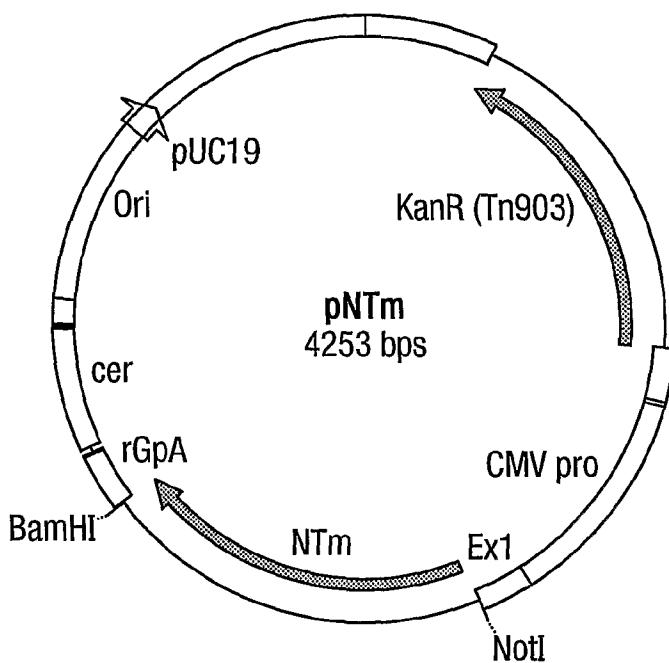


Fig.4.

Plasmid pNTm:



Sequence of insert:

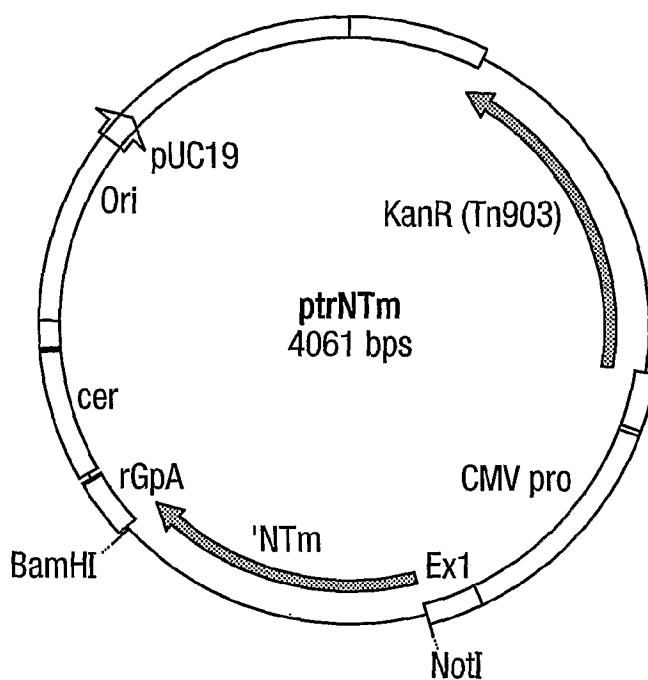
ATGGGTGGCAAGTGGTAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAAGAATGAGACGAGCTGA
 GCCAGCAGCAGATGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACAAGTACCAATA
 CAGCAGCTACCAATGCTGCTTGCCCTGGCTAGAACGCCAAAGAGGAGGAGGAGGAGGTTTCCAGTCACA
 CCTCAGGTACCTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTAAAGAAAAGGG
 GGGACTGGAAGGGCTAATTCACTCCCACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAG
 GCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCAGATATCCACTGACCTTGGATGGTGC
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 CCCTGTGAGCCTGCATGGAATGGATGACCCCTGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCTAG
 CATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAGAACTGCACTAGTGGCCAGTAGATCCT
 AGACTAGAGCCCTGGAAGCATTCAAGGAAGTCAGCCTAAAATGCTTGACCAATTGCTATTGTAAGGAGTG
 TTGCTTCATTGCCAAGTTGTTCATAACAGCTGCCTTAGGCATCTCTATGGCAGGAAGAAGCCGAGAC
 AGCGACGAAGACCTCTCAAGGCAGTCAGACTCATCAAGTTCTATCAAAGCAACCCACCTCCAAATCC
 AAAGGGAGCCGACAGGCCCGAAGGAATAA [SEQ ID NO: 51]

Amino acid sequence of antigen:

MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEE
 EEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLERGLIHSQRQRDILDLWIYHTQGYFPDWQNYT
 PGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDRLAFH
 HVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHQCVCFTAALGISYGRK
 KRRQRRRPPQGSQTHQVSLSKQPTSQSKGEPTGPKE [SEQ ID NO: 52]

Fig.5.

Plasmid ptrNTm:



Sequence of insert:

```

ATGGTGGGTTTCCAGTCACACCTCAGGTACCTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAG
CCACTTTAAAAGAAAAGGGGGACTGGAAGGGTAATTCACTCCAACGAAGACAAGATATCCTTGATC
TGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCAGATAT
CCACTGACCTTGGATGGTCTACAAGCTAGTACCAAGCTAGTACCGATTGAGCCAGATAAGTAGAAGAGGCCAATAAAGG
AGAGAACACCCAGCTTGTACACCCCTGTGAGCCTGCATGGAATGGATGACCTGAGAGAGAAGTGTAGAGT
GGAGGTTTGACAGCCGCTAGCATTCATCACGTGGCCGAGAGCTGCATCCGGAGTACTTCAAGAACCTGC
ACTAGTGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAAGTGTGTTGAC
CAATTGCTATTGAAAAAGTGTGCTTCATTGCAAGTTGTTCATAACAGCTGCCTAGGCATCTCCT
ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTCTATCA
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Amino acid sequence of antigen:

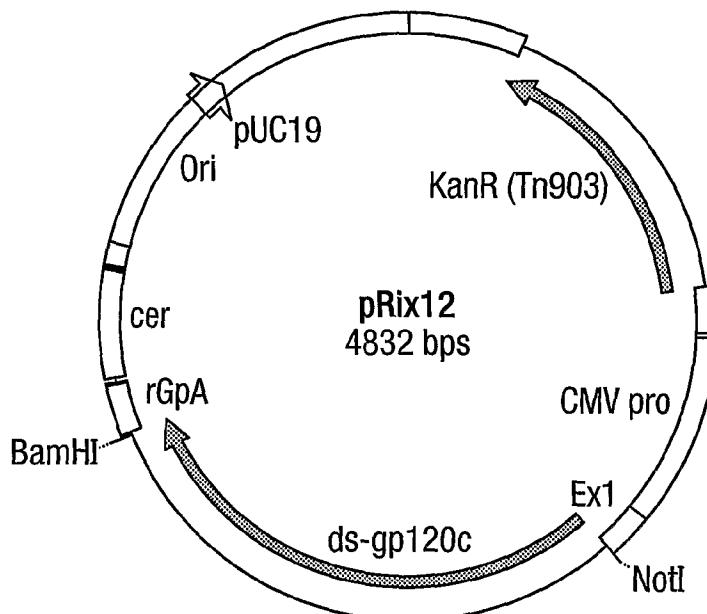
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MVGFPVTPQVPLRPMTYKAADVLSHFLKEKGGLIHSQRQDILDLWIYHTQGYFPDWQNYTPGPGVRY
PLTEFGWCYKLPVPVEPDKEEANKGENTSLHPVSLHGMDDPEREVLEWRFDLSRALHHVARELHPEYFKNC
TSEPVDPRLEPWKHPGSQPKTACTNCYCKKCFHCQVCFITAALGISYGRKKRRQRRPPQGSQTHQVSL
KQPTSQSKEPTGPKE [SEQ ID NO: 54]

```

Fig. 6.

Plasmid pRix12:



Sequence of insert:

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ATGGCCGAGCAGCTGTGGTCACCGTCACTACGGCGTGCGCTGTGGAAGGAGGCCACGACCACCTCTT
CTGGCGAGCAGCCAAGGCCTACGACACGGAAAGTCATAACGTGTGGCGACCGATGCTGCGTGCTA
CGGACCCCACCCCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGTCCCTGAAGCCCTGGCTGAAGCTGACGCC
TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCAACAGCAGTACCAACACCAGCAACGGCTGGA
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CAGAAGGAATACGCGCTGTTTATAATCTGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
CAAGACGACGCGTAATTCACTCATGCAACACAGCAGCGTCATGACGCAAGGCTGCCCAAGGTGT
CCTTCGAACCAATCCCGATCCATTACTGTGCCCCCTGCCGGATTCGCGATCCTCAAGTGTAAACAACAAGACC
TTCGACGGGAAGGGCCTGTGCACCAACGTCAAGCACGGTGAGTGCACCCATGGCATCCGCCCCGTGAG
CACCCAGCTGCTGTAACGGGTCCCTGGCTGAGGAGGGTGGTATCCGGTGGACAACCTTATGGACA
ACACCAAGACAATCATGTCAGCTGAACGAGTCTGTGGCGATTAACGTACCCGGCTAACAAACAACACC
CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTCTATGCCGCCGCAAGATCATGGCGACATCCGGCA
GGCCCATTGCAACCTCTCCCGGCCAGTGGAAATAACACCCCTGAAGCAGATCGTGTCAAGCTGAGAGAGC
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AACTGGGGGGCGAGTTCTTCACTGCGATACGACACAGCTCTCAACTCCACCTGGAACGGCACCGAGGG
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AGGTGGGAAAGGCCATGTATGCCCTTCACTGCGCTGCTCCAAACATCACCGGCCCTG
CTGCTCACCAAGAGACGGGGCACCGAGGGCAACGGCACGGAGAACGACAGGAGATCTTCAGGGCCGGCG
CGCGACATGAGGGATAACTGGCGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTGGCG
TGGCCCCACCCGGCCAAGCGCCGCGTCGTGAGAGATGA [SEQ ID NO: 55]

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Amino acid sequence of antigen:

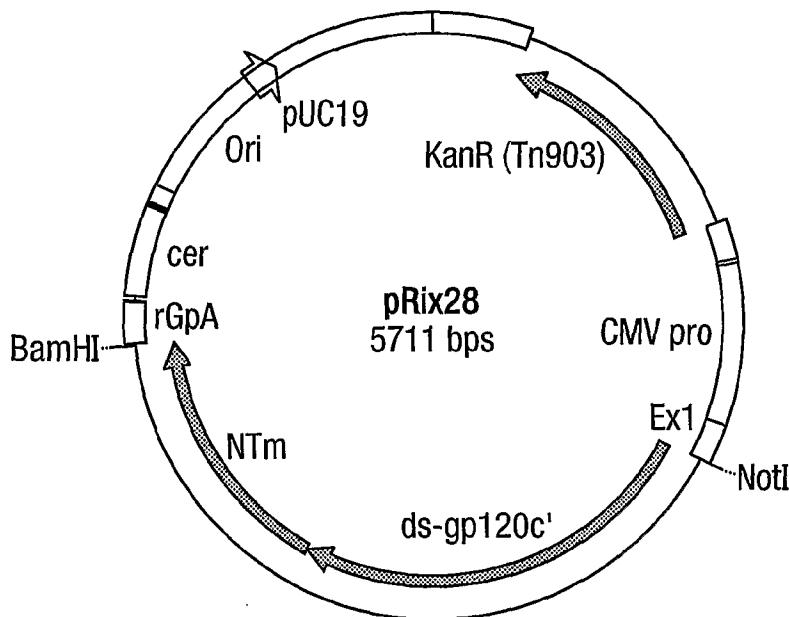
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QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNKNT
FDGKGLCTNVSTVQCTHGI RPV VSTQLLLNGSLAEEEVVI RSDNFMDNTKTII VQLNESVA IN CTRPNNT
RKGIHIGPGRAYAARKI IGD IRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
NC GGEFFYCDTTQLFNSTWNGTEGNNT EGNS TILPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
LLTRDGTEGNGTENETE IFRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQR [SEQ ID
NO: 56]

```

Fig. 7.

Plasmid pRix28:



Sequence of insert:

```

ATGGCCGAGCAGCTGTGGGTACCGTCACTACGGCGTGCCTGTGTGGAAAGGAGGCCACGACCACCCCTTT
CTGCGCGAGCGACGCCAAGGCCAACGACACGGAAAGTCATAACGTGTGGCGACCCATGCTTGCGTGCTTA
CGGACCCAACCCCCCAGGAGGTGGCTGGAAACGTCAGCGACTTCAACATGTGGAAAGAATAACATG
GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGTCCCTGAAGCCCTGCGTGAGCTGACGCC
TCTCTGGGTGACACTGGACTGTGACGACGTCAACACCACAGCACTACCACCAACAGCAACGGCTGG
CCGGAGAGATTGGAAAGGGCAGATCAAGAACTGCTCTTCAATATCACGACCTCGATCAGAGACAAGGTG
CAGAAGGAATACGGCTGTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCAAGAA
CAAGACGACGCGTAATTTCAGACTCATCACTGCAACAGCAGCGTCATGACGCAAGGCTGCCCAAGGTGT
CCTTCGAACCAATCCGATCCATTACTGTGCCCCCTGCCGGATTTCGCGATCTCAAGTGTAAACAACAAGACC
TTCGACGGGAAGGGCCTGTGCACCAACGTCAGGACGGTGCAGTGCACCCATGGCATCCGCCCCGTGAG
CACCCAGCTGCTGTAACGGGCTCCCTGGCTGAGGAGGAGGTGGTGAATCCGGTGGACAACCTCATGGACA
ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCTAACACAACACC
CGTAAGGGCATCCACATCGGCCCTGGACGGGCCTCTATGCCGCCGAAAGATCATGGCGACATCCGGCA
GGCCCATTGCAACCTCTCCCGGCCAGTGGAAATAACACCCCTGAAGCAGATCGTATCAAGCTGAGAGAGC
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AAGCACAAGAGGAGGAGGAGGTGGTTTCCAGTCACACCTCAGGTACCTTAAAGACCAATGACTTACAAG
GCAGCTGTAGATCTAGCCACTTTAAAAGAAAAGGGGGACTGGAAGGGCTAATTCACTCCAAACGAAG
ACAAGATATCCTGATCTGTGGATCTACCAACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAG
GGCCAGGGCTCAGATATCCACTGACCTTGGATGGCTACAAGCTAGTACCGAGTGGCAGATAAGGTA
GAAGAGGCCAATAAAGGAGAGAACACCAAGCTTGTACACCCCTGTGAGCCGCTAGCATTCACTACG
GAGAGAAGTGTAGAGTGGAGGTTGACAGCCGCTAGCATTCACTACGTTGGCCGAGAGCTGCATCCGG
AGTACTTCAAGAACTGCACTAGTGTAGCCAGTAGATCCTAGACTAGAGCCCTGGAAAGCATCCAGGAAGTCAG

```

Fig.7 (Cont.).

CCTAAAACGTGTTGACCAATTGCTATTGTAAGTGGCTTCATTGCCAAGTTGTTCATACAGC
TGCCTTAGGCATCTCTATGGCAGGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTC
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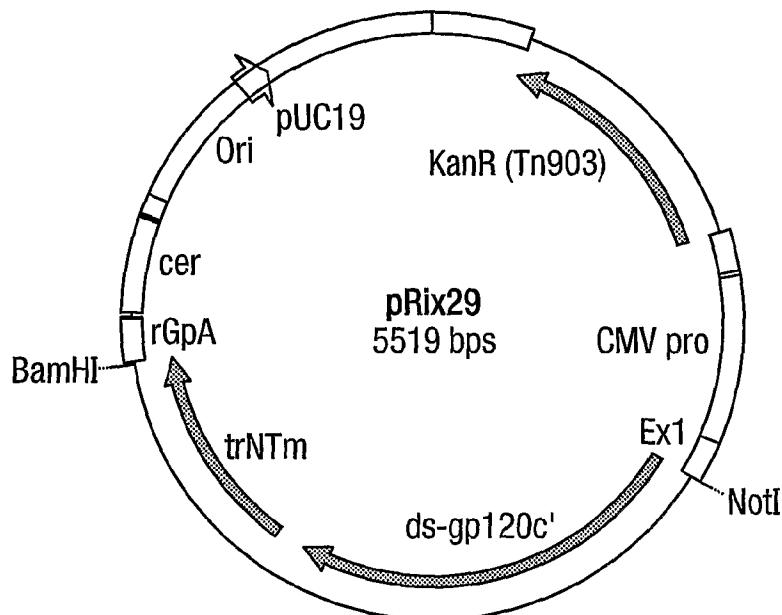
[SEQ ID NO: 57]

Amino acid sequence of antigen:

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QKEYALFYNLDVVPIDDDNATTKNKTRNFRЛИHCNSSVMTOACPKVSEPIPIHYCAPAGEFAILKCNKNT
FDGKGLCTNVSTVQCTH GIRPVVSTQLLNGSLAEEEVIRSDNFMDNTKTIIVQLNESVAINCTRPNNT
RKGIHIGPGRAYAARKIIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTICKFNQSSGGDPEIVRSF
NCGGEFFYCDTTQLFNSTWNGTEGNNTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
LLTRDGGETEGNGTENETEIFRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVQRMGGKWSKSSVV
GWPTVRERMRRRAEPAAADGVGAASRDLEKHGAITSNTAATNAACAWLEAQEEEVEGFPVTPQVPLRPMTYK
AAVDLSHFLKEKGGLEGLIHSQRQDILDLWIYHTQGYFPDWQNYTPPGPGVRYPLTFGWCYKLVPVEPDKV
EEANKGENTSLHPVSLHGMDPEREVLEWRFDSSLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQ
PKTACTNCYCKKCCFHQCQCFITAALGISYGRKKRRQRRPPQGSQTHQVSLSKQPTSQSKEPTGPKE
[SEQ ID NO: 58]

Fig.8.

Plasmid pRix29:



Sequence of insert:

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 CGGACCCCCAACCCCCCAGGAGGTGGTCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCATCCCTGAAGCCCTGCCTGAAGCTGAGCC
 TCTCTGCCTGACACTGGACTGTGACGACGTCAACACCAACAGCACTACCAACCCAGCAACGGCTGG
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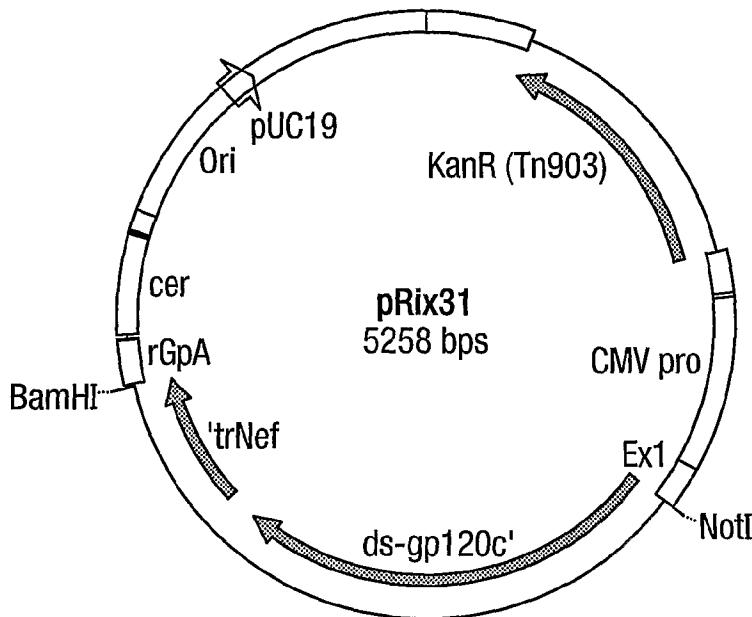
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Amino acid sequence of antigen:

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Fig. 9.

Plasmid pRix31:



Sequence of insert:

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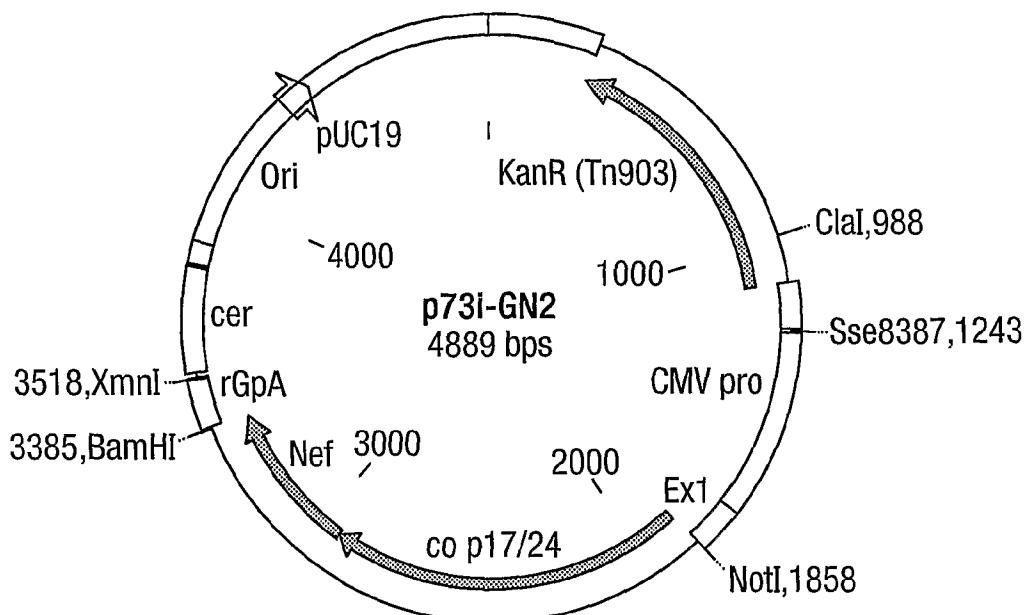
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Amino acid sequence of antigen:

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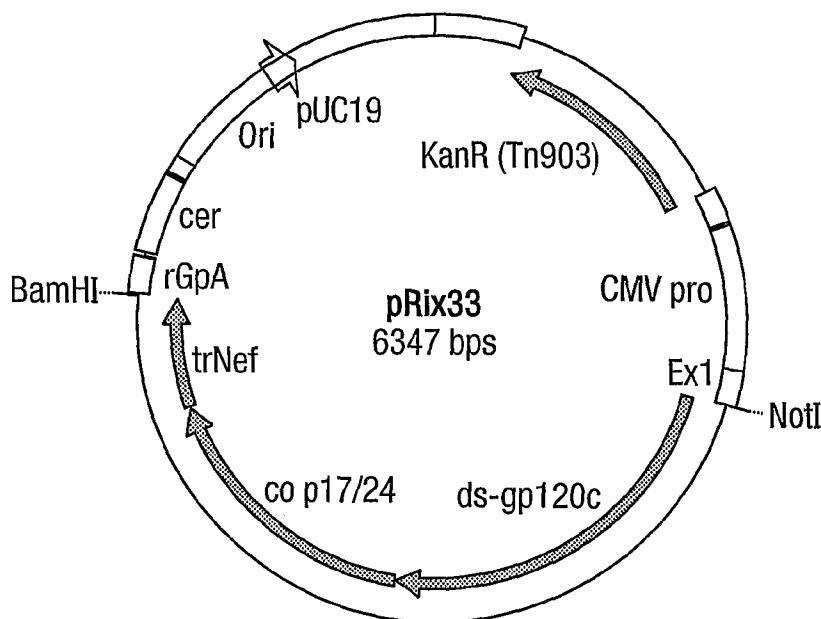
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Fig.11.

Plasmid pRix33:



Sequence of insert:

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Fig.11 (Cont).

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[SEQ ID NO: 64]

Amino acid sequence of antigen:

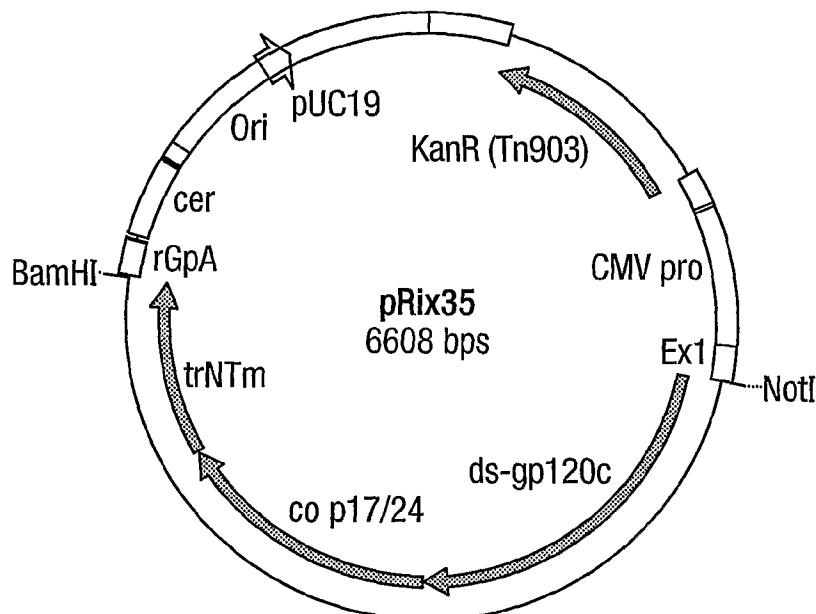
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[SEQ ID NO: 65]

Fig.12.

Plasmid pRix35:



Sequence of insert

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Fig.12 (Cont).

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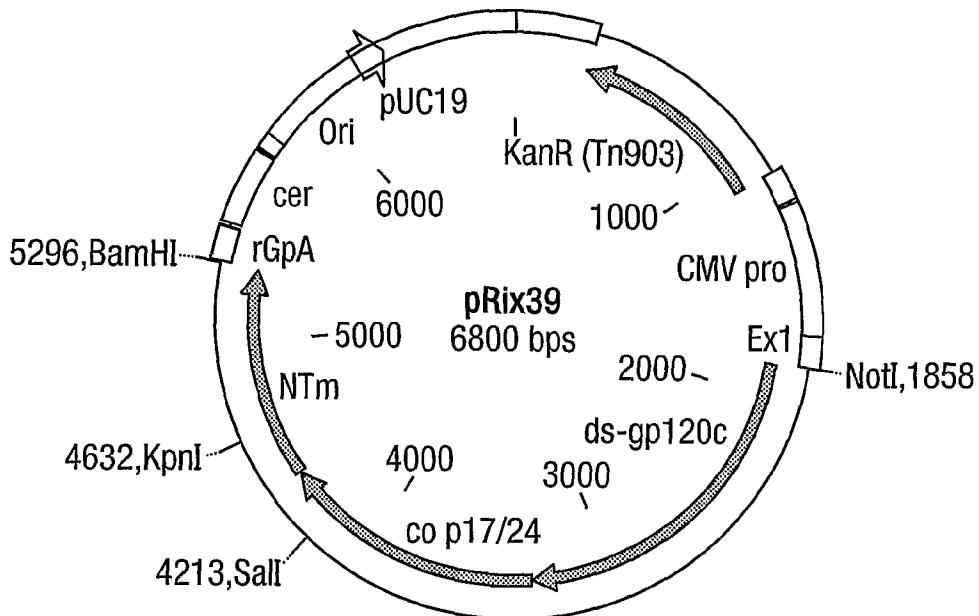
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Fig.13.



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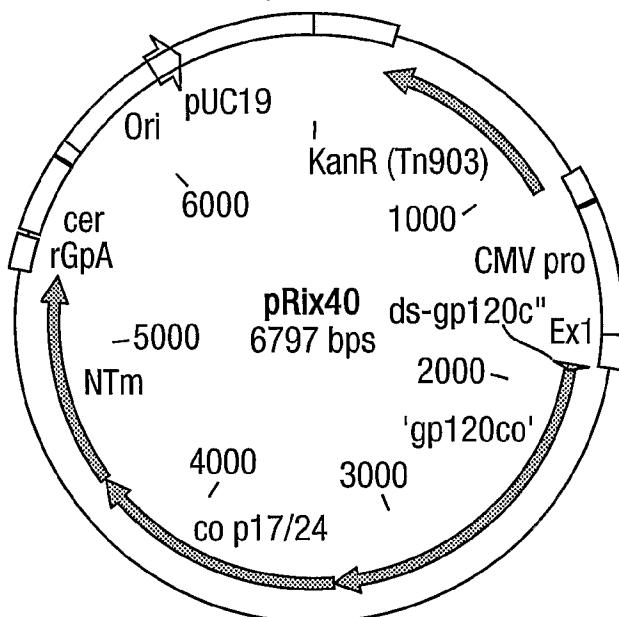
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 GGCCCATTGCAACCTCTCCCGGCCAGTGGAAATAACACCCCTGAAGCAGATCGTGTCAAGCTGAGAGAGC
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 ATGGATGACCAACAATCCTCCCATTCCAGTTGGAGAAATCTATAACGGTGGATATTCTGGTCTCAATA
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Fig.13 (Cont).

TACGTCGACCGGTTTATAAGACCCCTGCGAGCAGAGCAGGCCCTCTCAGGAGGTCAAAACTGGATGACGGA
GACACTCCTGGTACAGAACGCTAACCCCGACTGCAAAACAATCTGAAGGCACTAGGCCGGCTGCCACCC
TGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCCGGACACAAAGCCAGAGTGTGATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC
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CCAATGCTGCTTGTGCCCTGGCTAGAACGACAAGAGGAGGAGGAGGAGGTTTCCACTCACACCTCAGGTA
CCTTTAACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTAAAGAAAAGGGGGACTGGA
AGGGCTAATTCACTCCCAAGAACAGAACAGATATCCTGATCTGTGGATCTACCACACACAAGGCTACTTCC
CTGATTGGCAGAACTACACACCAGGGCAGGGTCAGATATCCACTGACCTTGGATGGTGTACAAGCTA
GTACCAAGTTGAGCCAGATAAGGTAGAACAGGCCATAAAGGAGAGAACACCAGCTTGTACACCCCTGTGAG
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CCCTGGAAAGCATCCAGGAAGTCAGCCTAAACTGTTGACCAATTGCTATTGTAAGGAGTGTGCTTCA
TTGCCAAGTTGTTCTATAACAGCTGCCTAGGCATCTCCTATGGCAGGAAGACAGCGAGACAGCGACGAA
GACCTCCTCAAGGCAGTCAGACTCATCAAGTTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGAG
CCGACAGGCCGAAGGAATAA [SEQ ID NO: 68]

Amino acid sequence of antigen:

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 LLTRDGGE NGNTENETEI FRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVQRM GARASVLSGG
 ELDRWEKIRLRPGGKKYKLKHIVWA RELERFAVNPGLETSEGCRCQI LGQLOPSLQTGSEELRSLYNTV
 ATLYCVHQRIEIKDTKEALDKIEEEQNKSKKQAQAAA DTGHSNQVSQNYPIVQNIQGQM VHQAIS PRTLN
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 YVDRFYKTLRAEQASQEVKNWMTETLLVQ NANPDCKTILKALGP AATL EEMMTACQGVGGPGHKARVLMGG
 KWSKSSVVGWPTV RERM RAEPAADGVGAASRDLEKH GAI TSSNTAATNAACAWL EAQEEEVGFPTPQV
 PLRPMTYKA AVDLSHFLKEKGGL EGHISQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTF GWCYKL
 VPVEPDKV EANK GENTSLLHPVSLHGMDDPERE VLEWRFD SRLAFHHVARELHPEYFKNC TSEPVD PRLE
 PWKHPGSQPKTACTNCYCKCCFH CQVC FITAALG ISYGRKKR RQRRP PQSQ THQV SLSK QPT SQSKGE
 PTGP K [SEQ ID NO: 69]

Fig. 14.**pRix40****DNA sequence of insert**

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 CGGACCCCACCCCCCAGGAGGTGGTGTGGAAACGTGACCGAGTACTCAACATGTGGAAGAATAACATG
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 TCTCTCGGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACAGCAACGGCTGGA
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 TTGACGGGAAGGGCCTGTGCACCAACGTCAGCACGGTGCACTGCACCCATGGCATCCGCCCCGTGAG
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 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACGTACCCGGCTAACAAACAACACC
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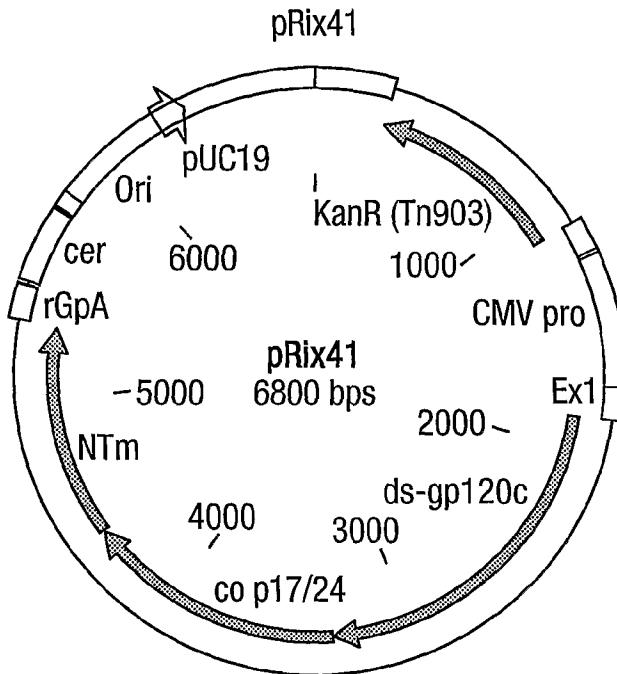
Fig.14 (Cont.).

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 AAATTGTTAGAATGTACTCTCCGACATCCATCCTGACATTAGACAGGGACCCAAAGAGCCTTTAGGGAT
 TACGTCGACCGGTTTATAAGACCCTGCGAGCAGAGCAGGCCCTCTCAGGAGGTAAAAACTGGATGACGGA
 GACACTCCTGGTACAGAACGCTAACCCGACTGCAAAACAATCTTGAAAGGCAGTAGGCCGGCTGCCACCC
 TGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGACACAAAGCCAGAGTGTGATGGGCAAG
 TGGTCAAAAAGTAGTGTGGTTGGATGGCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGA
 TGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACAAGTAGCAATAACAGCAGCTACCA
 ATGCTGCTTGTGCCCTGGCTAGAAGCACAAAGAGGAGGGAGGGTTTCCAGTCACACCTCAGGTACCT
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 CCAGTTGAGCCAGATAAGGTAGAAGAGGCAATAAGGAGAGAACACCAGCTGTTACACCTGTGAGCCT
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 TGGAAAGCATCCAGGAAGTCAGCCTAAACTGCTTGACCAATTGCTATTGTAAGGAGTGTGCTTCAATTG
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Aminoacid sequence of insert

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 RKGIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLQIVIKLREHFGNKTIFKNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNNT EGNSTITLPCRIKQIIINMWQEVGKAMYAPP IGGQIRCSSNITGL
 LLTRDGGE TEGNGTENETE IFRPGGGDMRDNRSELYKYKVVVKVEPLGVAPTRAKRRVVQRM GARASVLSGG
 ELDRWEKIRLRPGKKYKLHIVWA SRELERFAVNPGLLETSEGCRQILGQLQPSLQTGSEELRSLYNTV
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 YVDRFYKTLRAE QASQE VKNW MTETLLVQ NANPDCKT ILK ALGP AATI EEMM TACQGVGGPGH KARV LMGK
 WSKSSVVGPTVR ERMR RAEP AADGVGA ASR DLK EHG AITS SNTA ATNA AC AWL EA QEEE EVGF PVTPQVP
 LRPMTYKAAV DLSHFLKEKG GLEGLIHSQ RRQD ILDI WIYHTQGYFP DWQ NYTPGPGV RYPLTF GW CYKL V
 PVEPDKV EEEANK GENT SLLHPV S LHM DDP ERE VLEWRFD SRLA FH VARE LHPEYFKN CTSE PVD PR LEP
 WKHPGSQPKTACTNCYCKCC FHCQVC FITA ALG ISYGRK RRQ RRRP PQGS QTHQ VSLS KQ PTSQS KG EP
 TGPKE [SEQ ID NO: 71]

Fig.15.

**DNA sequence of insert**

ATGGCCGAGCAGCTGTGGGTACCGTCTACTACGGCGTGCCTGTGTGGAAGGAGGCCACGACCACCCCTTT
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 CGGACCCAACCCCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCAACAGCACTACCACCAACAGCAACGGCTGGA
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 CAGAAGGAATACGCGCTGTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCAAGAA
 CAAGACGACGCGTAATTCAGACTCATGCAACAGCAGCGTCACTGACGCAAGGCTGCCCAAGGTGT
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 GGCCCATTGCAACCTCTCCCGGCCAGTGGATAAACACCCCTGAAGCAGATCGTGTCAAGCTGAGAGAGC
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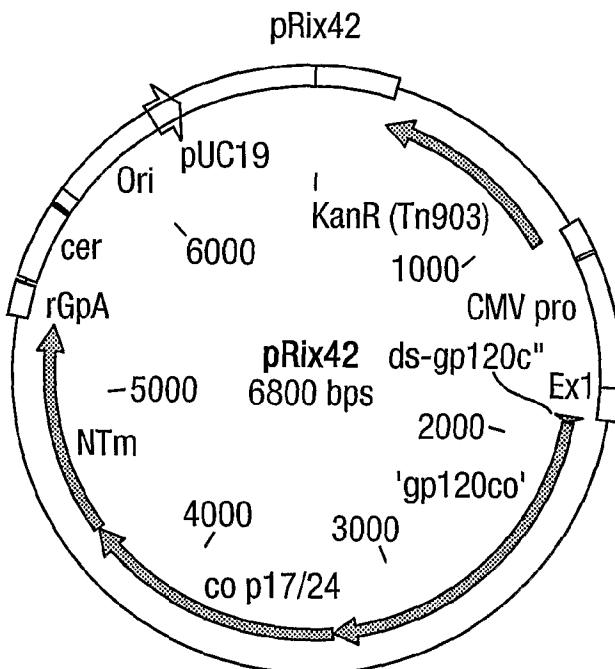
Fig.15 (Cont).

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Aminoacid sequence of insert

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 FDGKGLCTNVSTVQCTHGRPVSTQLLNGLAEEEVIRSDNFMDNTKTIIIVQLNESVAINC TRPNNT
 RKGIHIGPGRFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFQNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNNTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
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 AWKVVVEEKAFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQAAMQMLKETINEEEAEWDRVHPVHAGPIA
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 YVDRFYKTLRAEQASQEVKNWMTETLLVQNANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVLMGG
 KWSKSSVVGWPTVRERMRAEA PAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEEEVGFPVTPOV
 PLRPMTYKAADVDSLKEKGGLLEGHLHSQRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKL
 VPVEPDKVEEANKGENTSALHPVSLHGMDDPEREVLERWFDSRLAFHHVARELHPEYFKNCNSEPVDPRLE
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 PTGPKE [SEQ ID NO: 73]

Fig.16.

**DNA sequence of insert**

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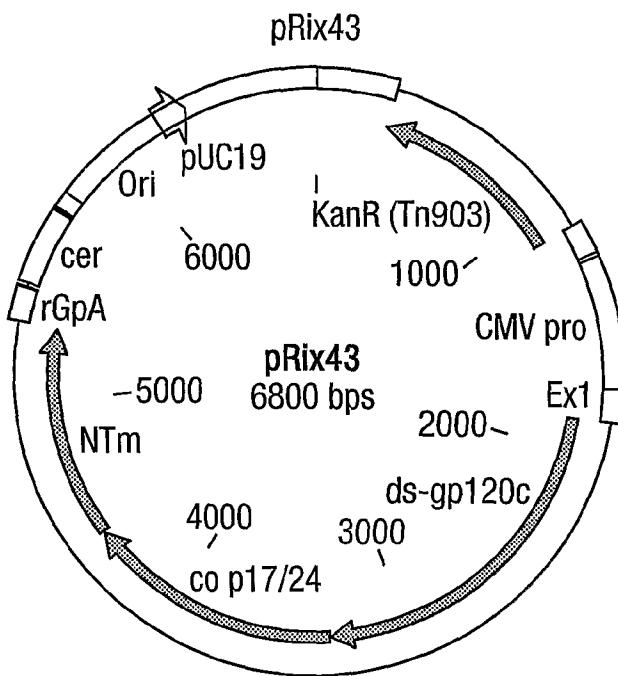
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Aminoacid sequence of insert

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 LLTRDGGETEGNGTENETEIFRPGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRVRVQRMGARASVLSGG
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 PLRPMTYKAADVLSHFLKEKGGLEGLIHSQRRODILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKL
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 PTGPKE [SEQ ID NO: 75]

Fig.17.

**DNA sequence of insert**

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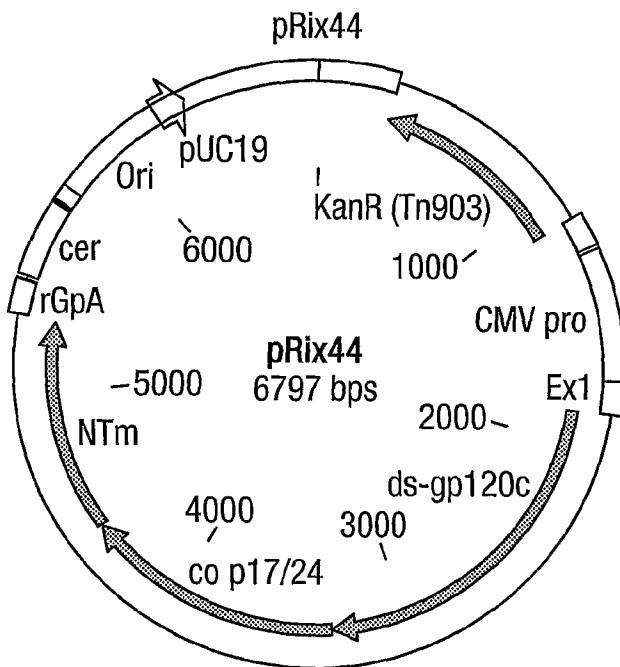
Fig.17 (Cont).

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 CCGACAGGCCGAAGGAATAA [SEQ ID NO: 76]

Aminoacid sequence of insert

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 PLRPMTYKAADVLSHFLKEKGGLEGLIHSQRQDILDWLWYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKL
 VPVEPDKVEEANKGENTSAAHPVSLHGMDPEREYLEWRFDSSLAFHHVARELHPEYFKNCTSEPVDPRL
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 PTGPKE [SEQ ID NO: 77]

Fig.18.

**DNA sequence of insert**

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CGGACCCAACCCCCAGGAGGTGGCTGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGCCCTGCGTGAAGCTGACGCC
TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCAACAGCAACGGCTGGA
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Fig.18 (Cont).

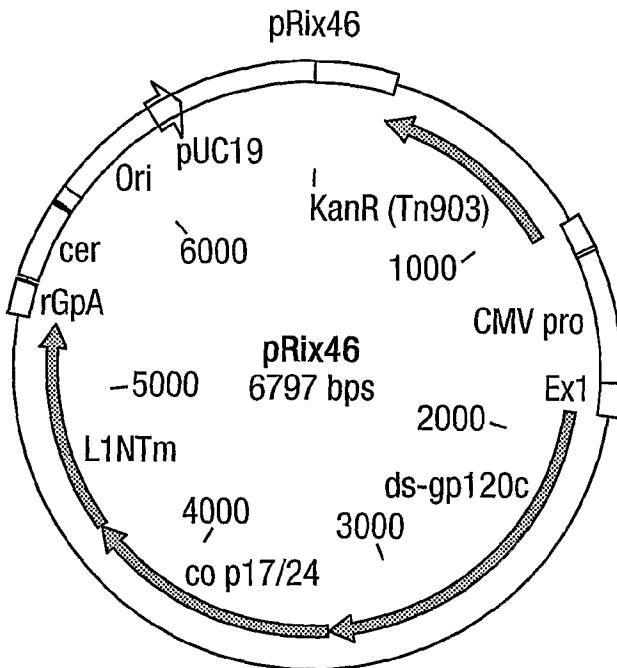
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Aminoacid sequence of insert

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 TGPKE [SEQ ID NO: 79]

30/64

Fig. 19.

**DNA sequence of insert**

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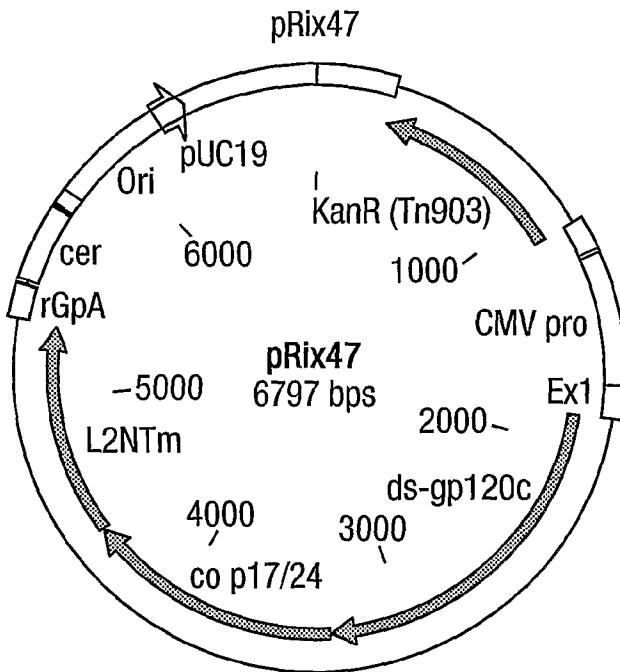
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Aminoacid sequence of insert

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 ELD RWEKIRLRPGKKYKLKHIVWASRELERFAVNPGLETSEGCRCQILGQLQPSLQTGSEELRSLYNTV
 ATLYCVHQ RIEIKDTKEALDKIEEQNKS KKAQQAADTGH SNQVSQNYPIVQNIQGQMVHQAI SPTLN
 AWVKVVEEKAFSPEVIPMFSA LSEGATPQDLNTMLNTVGGHQAAMQMLKETINEEAEWDRVHPVHAGPIA
 PGQMREPRGSDIAGTTSTLQEIQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSILD IRQGPKEPFRD
 YVDRFYKTLRAEQA S QEVKNWMTETLLVQ NANPDCKTILK AL GPAATLEEMMTACQGVGGPGH KARVLMGK
 WSKSSVVGWPTVRERM RRAEPAADGVGAASRDLEKHGAITSNTAATNAACAWLEAQEEE EVGF PVTPQVP
 LRPMTYKAAV DLSHFLKEKG GLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW CYKLV
 PVEPDKV EEA NK GENT S ALHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNCTSEPVDP RLEP
 WKH PGSQPKTACTNCYCKCCFHCQVC FITAALG ISYGRKKRQR RRPPQGSQTHQV SLSKQPTS QSKGE P
 TGPKE [SEQ ID NO: 81]

Fig.20.

**DNA sequence of insert**

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CGGACCCCACCCCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
GTGGATCAGATGCACTGAGGACATCATCTCTGTGGGACCACTCCCTGAAGCCCTGCGTGAAGCTGACGCC
TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACAGCAACGGCTGGA
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GGAGCAAAACAAGAGCAAGAAGAAGGCCAGCAGGAGCTGCTGACACTGGGATAGCAACAGGTATCAC
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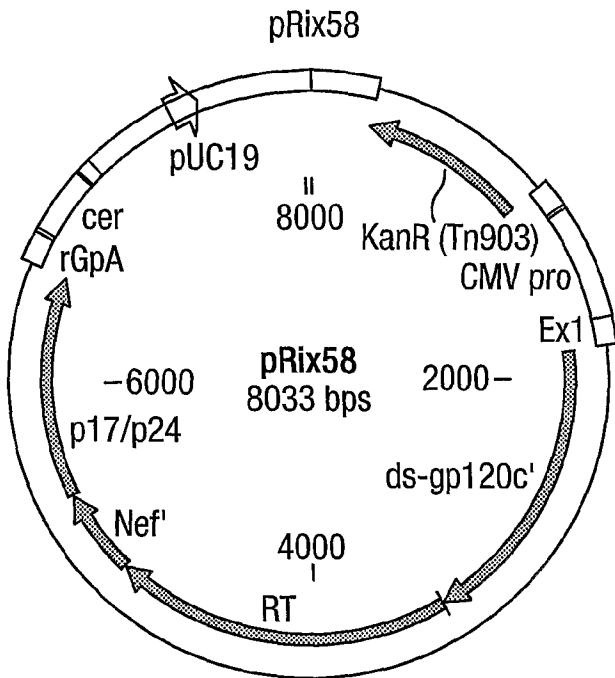
Fig.20 (Cont).

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 ATGGATGACCAACAATCCTCCCCTCCAGTTGGAGAAATCTATAAACGGTGGATCATTCTCGGTCTCAATA
 AAATTGTTAGAATGTACTCTCCGACATCCATCCTGACATTAGACAGGGACCCAAAGAGCCTTTAGGGAT
 TACGTGACCGGTTTATAAGACCCCTGCGAGCAGAGCAGGCCCTCAGGAGGTAAAAACTGGATGACGGA
 GACACTCCTGGTACAGAACGTAACCCGACTGCAAAACAATCTGAAGGCCTAGGGCCGGCTGCCACCC
 TGGAAAGAGATGATGACCGCCTGTCAAGGAGTAGGGGGACACAAAGCCAGAGTGTTGATGGGCAAG
 TGGTCAAAAAGTAGTGTGGTGGATGGCCTACTGTAAGGAAAGAATGAGACGAGCTGAGCAGCAGCAGA
 TGGGGTGGGAGCAGCATCTGAGACCTGGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCA
 ATGCTGCTTGTGCTGGCTAGAACAGCACAAAGAGGAGGAGGAGGTGGGTTTCCAGTCACACCTCAGGTACCT
 TTAAGACCAATGACTTACAAGGCAGCTGAGATCTTAGCCACTTTAAAAGAAAAGGGGGGACTGGAAGG
 GCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTCCCTG
 ATTGGCAGAACTACACACCAGGGCCAGGGTCAGATATCCACTGACCTTGGATGGTGCCTACAAGCTAGTA
 CCAGTTGAGCCAGATAAGGTAGAACAGGCCAATAAAGGAGAGAACACCAGCTTGGCACACCCCTGTGAGCCT
 GCATGGAATGGATGACCTGAGAGAGAAGTGTAGAGTGGAGGTTGACAGCCGCTAGCATTTCATCAGC
 TGGCCCGAGAGCTGCATCCGGAGTACTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTAGAGCCC
 TGGAAAGCATCCAGGAAGTCAGCCTAAACTGCTTGTACCAATTGCTATTGTAAGGAGTGTGCTTTCATTG
 CCAAGTTGTTCTACACAGCTGCCTAGGCATCTCTATGGCAGGAAGAGCGGAGACAGCGACGAAGAC
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 ACAGGCCGAAGGAATAA [SEQ ID NO: 82]

Aminoacid sequence of insert

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 PVEPDKVEEANKGENTS LAHPVSLHGMDDPEREVLEWRFDSSLAFHHVARELHPEYFKNCTSEPVDPRELP
 WKHPGSQPKTACTNCYCKKCCFHQCVCFITAALGISYGRKKRRQRRPPQGSQTHQVSLSKQPTSQSKEP
 TGPKE [SEQ ID NO: 83]

Fig.21.

**DNA sequence of insert**

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CGGACCCAACCCCCAGGAGGTGGTCTGGAAACGTGACCGAGTACTTCACATGTGGAAGAATAACATG
GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCACTCCCTGAAGCCCTGCGTAAGCTGACGCC
TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCAACAGCAACGGCTGGA
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GGCTGGAAGGGCTCTCCGCCATTTCAGAGCTCCATGACCAAGATCCTGGAGCCGTTCGGAAGCAGAA
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```

Fig.21 (Cont I).

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 CCCCTCACGGAGGAAGCCAGCTCGAGCTGGCTGAGAACCGGGAGATCCTGAAGGAGGCCGTGACGGCGT
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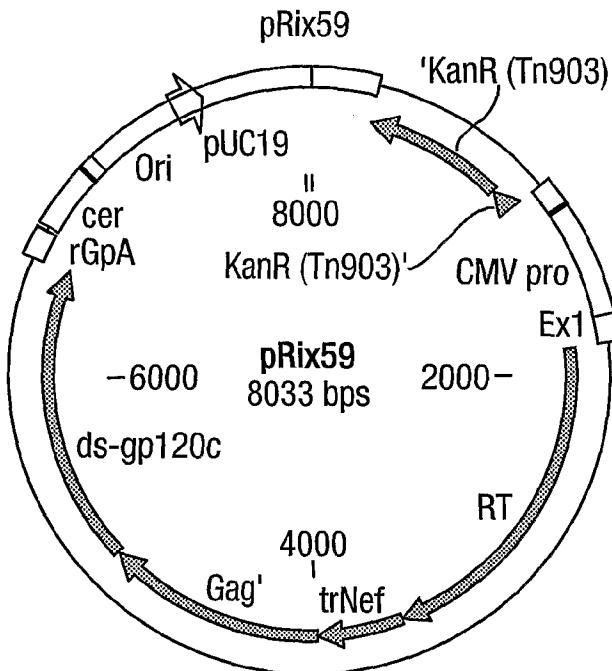
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 VKQLTEAVQKITTESIVIWGKTPFKLPIQKETWETWWTEYQWQATWIPEWEFVNTPPLVKLWYQLEKEPIV
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Fig.21 (Cont II).

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NO: 85]

Fig.22.

**DNA sequence of insert**

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GGTGGACCGAATATTGGCAGGCCACCTGGATTCCCGAGTGGAGTCTGTGAATACACCTCTGGTGAAG
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```

Fig.22 (Cont I).

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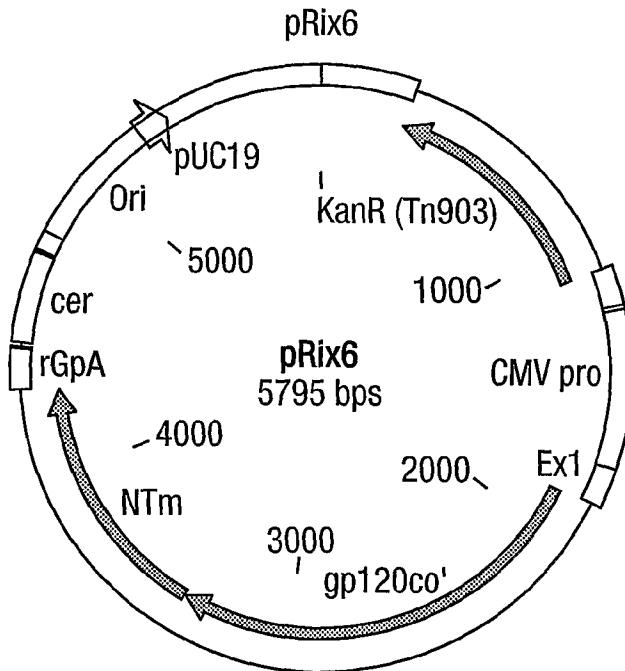
Aminoacid sequence of insert

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 WPAHKGIGGNEQVDKLV
 VSAGIRKVL
 MVGF
 PV
 TPQVPLRPMTYKA
 A
 VDLSHFLKE
 KGGLEG
 GLIHSQR
 RQDIL
 DLWIYHTQGY
 FPDWQNY
 TPGPGV
 RYPL
 TFGW
 CYKLP
 VEP
 PDK
 VEE
 ANKG
 ENTS
 LL
 HPV
 SL
 HGMD
 DP
 PER
 E
 VLE
 WR
 FDS
 RLA
 FH
 VARE
 LH
 PEY
 FKNC
 MGARAS
 VLSGG
 ELD
 RWE
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 PGK
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 YKL
 KH
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 LER
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Fig.22 (Cont II).

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NO: 87]

Fig.23.

**DNA sequence of insert**

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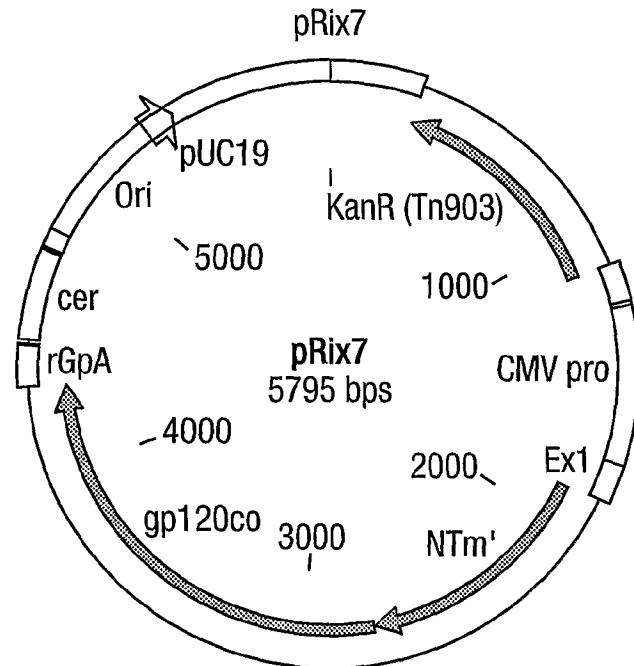
Fig.23 (Cont.).

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 GTTTCATAACAGCTGCCTAGGCATCTCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAA
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 GAAGGAATAA [SEQ ID NO: 88]

Aminoacid sequence of insert

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 CPKVSFEPPIPIHYCAPAGFAILKCNNKTFDGKGLCTNVSTVQCTHGIRPVVSTQLLNGSLAEEEVVIRSD
 NFMDNTKTIIVQLNESVAINCTRPNNNTRKGHIIGPGRAFYAARKIIGDIRQAHCLNSRAQWNNTLKQIVI
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 EPLGVAPTRAKRRVVQRMGGKWSKSSVVGWPTVRERMRAEAEPADGVGAASRDLEKHGAITSSNTAATNA
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 ELHPEYFKNCTSEPVDRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITAALGISYGRKKRRQRRPPQ
 GSQTHQVSLSKQPTSQSKEPTGPKE [SEQ ID NO: 89]

Fig.24.

**DNA sequence of insert**

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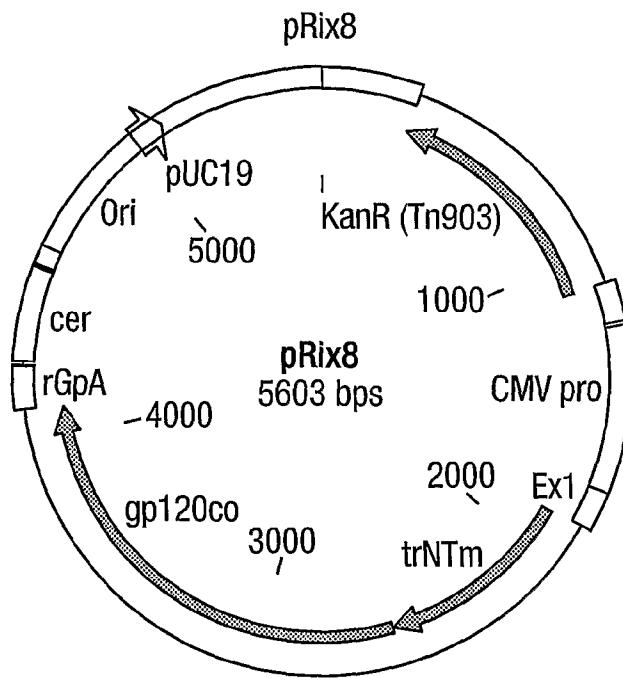
Fig.24 (Cont).

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GCAGAGATGA [SEQ ID NO: 90]

Aminoacid sequence of insert

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LYKYKVVKVEPLGVAPTRAKRRVVQR [SEQ ID NO: 91]

Fig.25.

**DNA sequence of insert**

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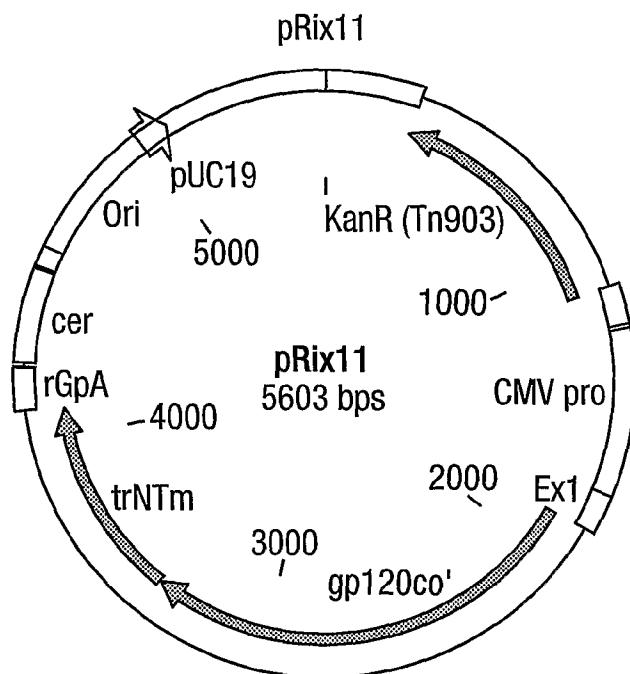
Fig.25 (Cont.).

AGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGCGTGGCCCCAC
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Aminoacid sequence of insert

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NLSRAQWNNTLKQIVIKLREHFGNKTIFNQSSGDPEIVRHSFNCGGEFFYCDTTQLFNSTWNGTEGNNT
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Fig.26.

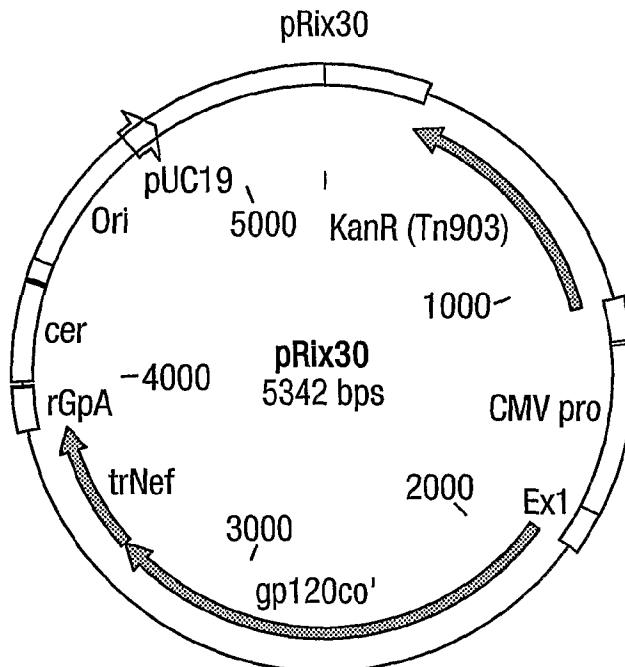


DNA sequence of insert

Aminoacid sequence of insert

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Fig.27.



DNA sequence of insert

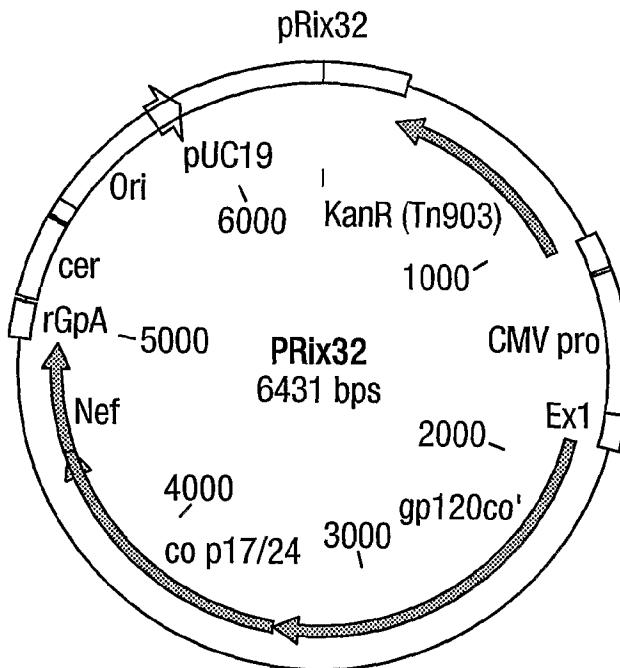
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 TCAGGCCCGCGCGACATGAGGGATAACTGGCGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGGT
 GAGCCGCTGGCGTGGCCCCACCCGGCCAAGGCCGCGTCGTGAGAGAATGGTGGTTTCCAGTCAC
 ACCTCAGGTACCTTAAGACCAATGACTTACAAGGAGCTGTAGATCTTAGCCACTTTAAAAGAAAAGG
 GGGGACTGGAAGGGCTAATTCACTCCAAAGAAGACAAGATATCCTGATCTGTGGATCTACCACACACAA
 GGCTACTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCAGATATCCACTGACCTTGGATGGT
 CTACAAGCTAGTACCAAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAGGAGAGAACACCAGCTGT
 ACCCTGTGAGCCTGCAATGGAATGGATGACCTGAGAGAGAGATGTTAGAGTGGAGGTTGACAGCCGCTA
 GCATTTCATCAGTGGCCCAGAGCTGCATCCGGAGTACTTCAAGAACTGCTAA [SEQ ID NO: 95]

Fig.27 (Cont).

Aminoacid sequence of insert

MKVKE TRK NYQHLW RWG TMLLG MLMIC SAAEQL WVT VYY GVPV WKE ATT LFC AS DAKAY DTE VHNV WATH
ACVPTDPN PQE VV LGNV TEY FNMWKNNMVDQM HEDI IISLWDQSLKPCVKLTPLCVTLDCDDVN TTNSTTT
SNGWTGEIRKGEIKNC SFN ITTSIRD KVQKEY ALFYNLDV VPID DDNATT K NKTRNFR LIHC NSSVMTQA
CPKVS F EPIPIHY CAPAGFA ILK CNNKTFDGKGLCTNV STVQ CTHGIRPVV STQ LLLNGSLAEEEVVIRSD
NFMDNTKT IIIVQLNE SVAINC TRPN NNTRKG I HIGP GRAFY AARKI IGD IRQAH CNLSRA QWN NTLK QIVI
KLREHFGNKT I KFNQSSGGDPEIVRHSFNC GGEFFYCDTTQLFNSTWNGTEGNNT EGNS TITLPCRIKQII
NMWQEVGKAMYAPP IGGQI RCSSN ITGLLLTRDGGTEGNGT ENETE I FRPGGGDMRD NWRS ELYKYKVV KV
EPLGVAPTRAKRRVVQRMVGFPVTPQVPLRPMTYKA AVDL SHFLKE KGGLE GLIHSQR RQDILD LWIYHTQ
GYFPDWQNYTPGPGVRYPLTFGWCYKLV PVEPDKVEEANKGENTSLLHPVSLHGMD DPEREVLEWRFD SRL
AFHHVARELHPEYFKNC [SEQ ID NO: 96]

Fig.28.



DNA sequence of insert

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GCTTGCGTGCCTACGGACCCAACCCCCAGGAGGTGGTGTGGAAACGTGACCGAGTACTCAACATGTG
GAAGAATAACATGGTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGTCCCTGAAGCCCTGCG
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AGCAACGGCTGGACCGGAGAGATTGGAAGGGCGAGATCAAGAAACTGCTCCTTAATATCACGACCTCGAT
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CCACCAAGAACAGACGACGCTAATTCAGACTCATTCACTGCAACAGCAGCGTCATGACGCAGGCC
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TAACAACAACACCGTAAGGGCATCCACATCGGGCTGGACGGGCTTCTATGCGCCCGCAAGATCATCG
GCGACATCCGGCAGGCCATTGCAACCTCTCCCGCCAGTGGAAATAACACCCCTGAAGCAGATCGTGATC
AAGCTGAGAGAGCACTTGGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGAGACCCCGAGATCGT
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ACGGCACCGAGGGCAACAACACAGAGGGAAACTCCACTATCACCCTCCCTGCCATCAAGCAGATCATC
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CATCACCGGCCTGCTGTCACCAGAGACGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCT
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GAGGGATGTCGCCAGATCCTGGGCAATTGCAACGCTCCAGACCGGGAGTGAAGAGCTGAGGTCTT
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ACAAAATGAGGAGGAGCAAAACAAGAGCAAGAAGAAGGCCAGCAGGGCAGCTGCTGACACTGGGCAATAGC
AACCAGGTATCACAGAACTATCCTATTGTCCAAAACATTCAAGGGCCAGATGGTTCATCAGGCCATCAGGCC
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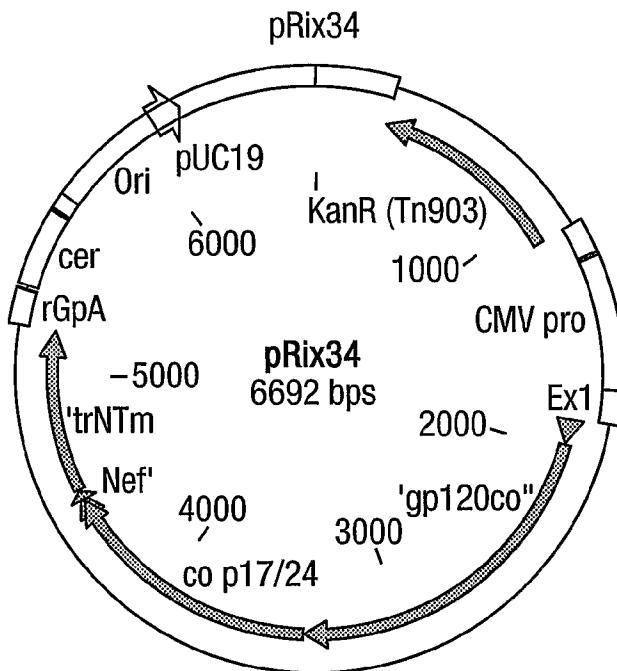
Fig.28 (Cont).

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 CCGGCTGCCACCCCTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGGCGACACAAAGCCAGAGT
 GTTGATGGTGGTTTCCAGTCACACCTCAGGTACCTTAAGACCAATGACTTACAAGGCAGCTGTAGATC
 TTAGCCACTTTAAAAGAAAAGGGGGACTGGAAGGGCTAATTCACTCCAAAGAACAGAACAGATATCCTT
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 GAGTGGAGGTTGACAGCCGCTAGCATTCATCACGTGGCCGAGAGCTGCATCCGGAGTACTTCAAGAA
 CTGCTGA [SEQ ID NO: 97]

Aminoacid sequence of insert

MKVKETRKNYQHLWRWGTMLLGMLMCSAAEQLWVTVYYGVPVWEATTTLFCASDAKAYDTEVHNWATH
 ACVPTDPNPQEVLGNVTEYFNMWKNNMVDQMHEDEIISLWDQSLKPCVKLTPLCVTLDCDDVNNTNSTTT
 SNGWTGEIRKGEIKNCFSNITTSIRDKVQKEYALFYNLDDVPIDDDNATTKNKTRNFRЛИHCNSSVMTQA
 CPKVSFEPIPIHYCAPAGFAILKCNKTFDGKGLCTNVSTQCTHIGRPVYSTQLLNGSLAEEEVVIRSD
 NFMDNTKTIIVQLNESVAINCTRPNNNTRKGHIHGPGRAYAARKIIIGDIRQAHCNLSRAQWNNTLKQIVI
 KLREHFGNKTIFKNQSSGGDPEIVRHNSFNCGGEFFYCDTQLFNSTWNGTEGNNTENEGNSTITLPCRIKQII
 NMWQEVGKAMYAPPPIGGQIRCSSNITGLLLTRDGGTEGNGTENETEIFRPGGDMRDNWRSLEYKYKVVKV
 EPLGVAPTRAKRRVQRMGARASVLSGGELDRWEKIRLREGKKYKLKHIVWASRELERFAVNPGLETS
 EGCRQILGQLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEQNKSKKKAQQAAADTGH
 NQVSQNYPIVQNIQGQMVHQAIISPRTLNAWVKVVEEKAFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQA
 AMQMLKETINEEAEWDRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEIQIGWMTNNPPIPVGEIYKRWII
 LGLNKIVRMYSPTSILDIROGPKEPFRDYVDRFYKTLRAEQASQEVKNWMETLLVQANAPDCKTILKALG
 PAATLEEMMTACQGVGGPGHKARVLMVGFPTPQVPLRPMTYKAAVDLSHFLKEKGGLLEGLIHSQRRODIL
 DLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLPVVEPDKVEEANKGENTSLHPVSLHGMDDPEREV
 EWRFDSRLAFHHVARELHPEYFKNC [SEQ ID NO: 98]

Fig.29.

**DNA sequence of insert**

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CGACCACCCCTCTCTGCAGCAGGCCAACGGCTACGACACGGAAAGTCATAACGTGTGGCGACCGCAT
GCTTGCCTGCCTACGGACCCAAACCCCCAGGAGGTGGTGTGGAAACGTGACCGAGTACTTCAACATGTG
GAAGAATAACATGGTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAAGCTGAAGCCCTGCG
TGAAGCTGACGCCCTCTGCCTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCC
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CCACCAAGAACAGACGACGCGTAATTCACTCATCAGACGCGTATGACGAGGCC
TGCCCCAAGGTGTCTCGAACCAATCCGATCCATTACTGTGCCCCCTGCCGGATTCGCGATCCTCAAGTG
TAACAAACAAGACCTTCGACGGGAAGGGCTGTGCACCAACGTCAAGCACGGTGAGTGCACCCATGGCATCC
GCCCGCTGTGAGCACCCAGCTGCTGTAACGGGTCCCTGGCTGAGGAGGGAGGTGGTATCCGGTGGAC
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Fig.29 (Cont.).

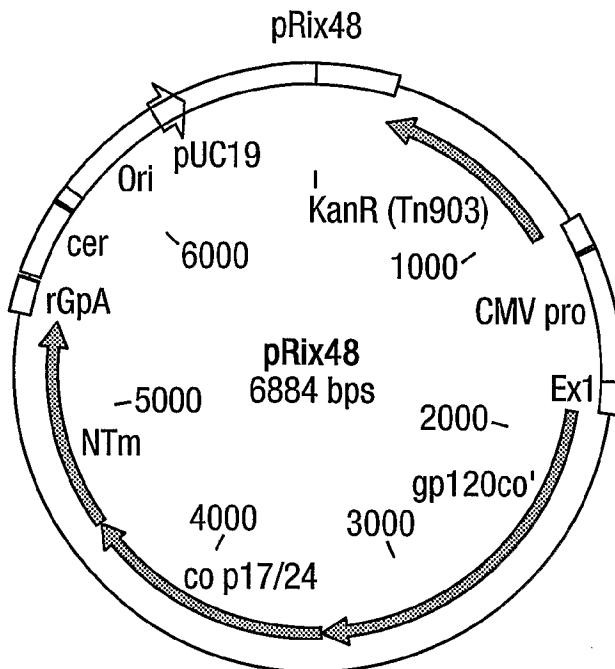
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 TCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCTCAAGGAGTCAGACTCATCAAGTTCT
 ATCAAAGCAACCCACCTCCAATCAAAGGGAGCCGACAGGCCGAAGGAATAA [SEQ ID NO: 99]

Aminoacid sequence of insert

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 ACVPTDPNPQEVLGNVTEYFNWKNNMVDQMHEIDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTT
 SNGWTGEIRKGEIKNSFNITTSIRDVKQKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHNSSSVMTQA
 CPKVSFEPPIHYCAPAGFAILKCNNKTFDGKGLCTNVSTVQCTHIGRPVVSTQLLNGSLAEEEVIRSD
 NFMDNTKTIIVQLNESVAINCTPNNNTRKGHIHGPGRFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVI
 KLREHFGNKTIFNQSSGGDEIWRHSFNCGGEFFYCDTTQLFNSTWNGTEGNNTEGNSTITLPCRRIKQII
 NMWQEVGKAMYAPPIGGQIRCSSNITGLLTRDGGTEGNGTENETEIFRPGGGDMRDNWRSLEYKYKVVK
 EPLGVAPTRAKRRVVQRMGARASVLSGEGELDRWEIRLRPGKKYKLKHIVWASRELERFAVNPGLETS
 EGCRQILGQLQPSIQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNKSKKAAQQAAADTGH
 NQVSQNYPIVQNIQGQMVKHQAISPRTLNAWVKVVEEKAFSPEVIPMFALSEGATPQDLNTMLNTVGGHQA
 AMQMLKETINEEAEWDRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEIQIGWMTNNPPIPVG
 GEIYKRWII
 LGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKNWMTEL
 LLVQNANPDCKTILKALG
 PAATLEEMMTACQGVGGPGHKARVLMVGFVTPQVPLRPMTYKA
 AAVDLSHFLKEKGGLEGLIHSQRQRQDIL
 DLWIYHTQGYFPDWQNYTPGPVRYPLTFGWCYKLPV
 VEPDKVEEANKGENTSLHPVSLHGMDDPEREVL
 EWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRL
 EPWPKHGPSQPKTACTNCYCKKCCFHQCVC
 FITAALGI
 SYGRKKRRQQRRPPQGSQTHQVSLSKQPTSQS
 KGEPTGPKE [SEQ ID NO: 100]

53/64

Fig.30.

**DNA sequence of insert**

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ATGAAGGTCAAGGAGACCAGAAAGAACTACCAGCATCTGGCGCTGGGCACCATGCTCCTGGGAATGCT
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TGAAGCTGACGCCCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACACC
AGCAACGGCTGGGACGGAGAGATTGGAAGGGCAGAGATCAAGAACTGCTCCTCAATATCACGACCTCGAT
CAGAGACAAGGTGAGCAGAAATACGCGCTGTTTAATCTCGATGTGGTCCCCATCGACGACGACAATG
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GCCCGTCGTGAGCACCCAGCTGCTGAACGGGTCCTGGCTGAGGAGGGTGGTATCCGGTGGAC
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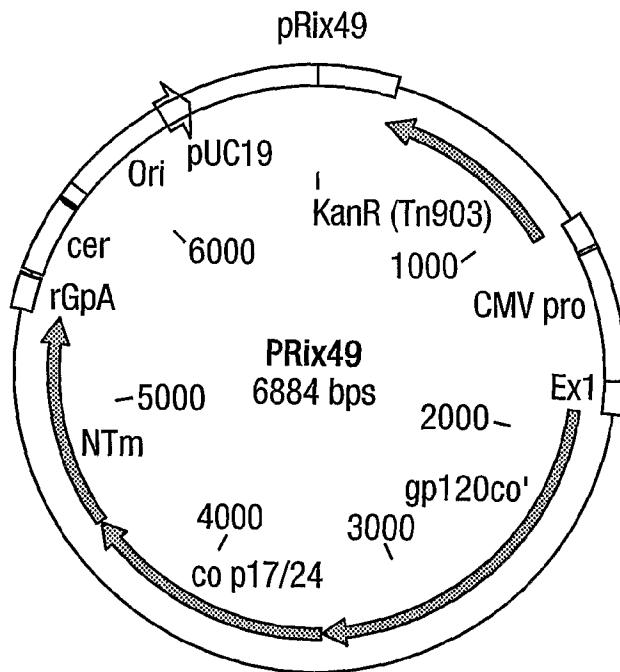
Fig.30 (Cont.).

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 ATCCAAAGGGGAGCCGACAGGCCGAAGGAATAA [SEQ ID NO: 101]

Aminoacid sequence of insert

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 CPKVSFEPPIHYCAPAGFAILKCNNKTFDGKGLCTNVSTVQCTHGIRPVVSTQLLNGSLAEEEVIRSD
 NFMDNTKTIIIVQLNESVAINCTRPNNTRKGIIHIGPGRAYAARKIIGDIRQAHNLSRAQWNNTLKQIVI
 KLREHFGNKTIFNFNQSSGGDPEIVRHSFNCGGEFFYCDTTQLFNSTWNGTEGNNTEGNSTITLPCRIKQII
 NMWQEVGKAMYAPPIGGQIRCSSNITGLLLTDGGTEGNGETENEIIFRPGGDMRDNRSELYKYKVVKV
 EPLGVAPTRAKRRVVQRMGARASVLSGGELDRWEKIRLRPGGKKYKLKHIVWASRELERFAVNPGLETS
 EGCRQILGQLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKDTKEALDKIEEEQNSKKKAQQAAADTGH
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 AMQMLKETINEEAEWDRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEIQIGWMNNPPIPVG
 GEIYKRWII LGLNKIVRMYSPSTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKNWMTETLLV
 QNANPDCKTILKALG PAATLEEMMTACQGVGGPGHKARVLMGGWSKSSVVGWPVRERMRAEPAADGV
 GAASRDLEKHGAITSS NTAATNAACAWLEAQEEEVEGFPVTPQVPLRPMTYKAAVDSLHFLKEGG
 LEGLIIHSQRQDILDLWIYHT QGYFPDWQNYTPGPGVRYPLTFGWCYKLPVPEPKV
 EANKGENTSLHPVSLHGMDDPEREVLEWRFDSR LAFHHVARELHPEYFKNCTSEPVDP
 RLEPWKHPGSQPKTACTNCYCKCCFHQCVCFITAALGISYGRK
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Fig.31.

**DNA sequence of insert**

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Fig.31 (Cont.).

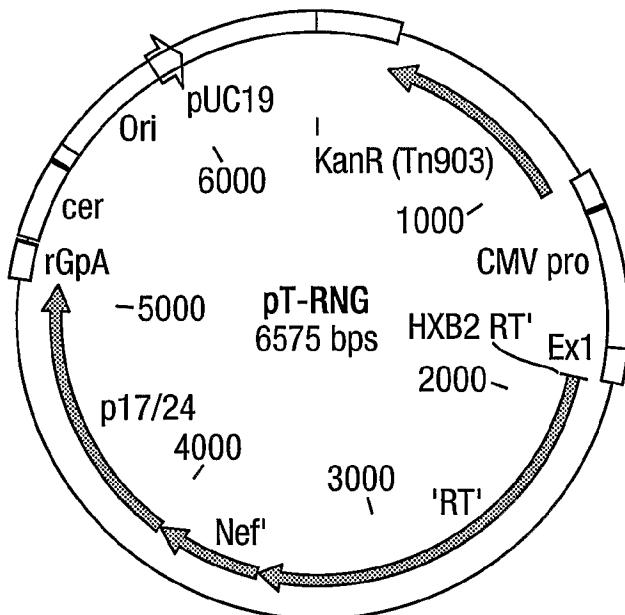
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Aminoacid sequence of insert

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Fig.32.

pT-RNG



DNA sequence of insert:

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Fig.32 (Cont.).

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Amino acid sequence of insert:

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VL [SEQ ID NO: 89]

Fig.33. A schematic representation of the constructs and associated expression data is shown below

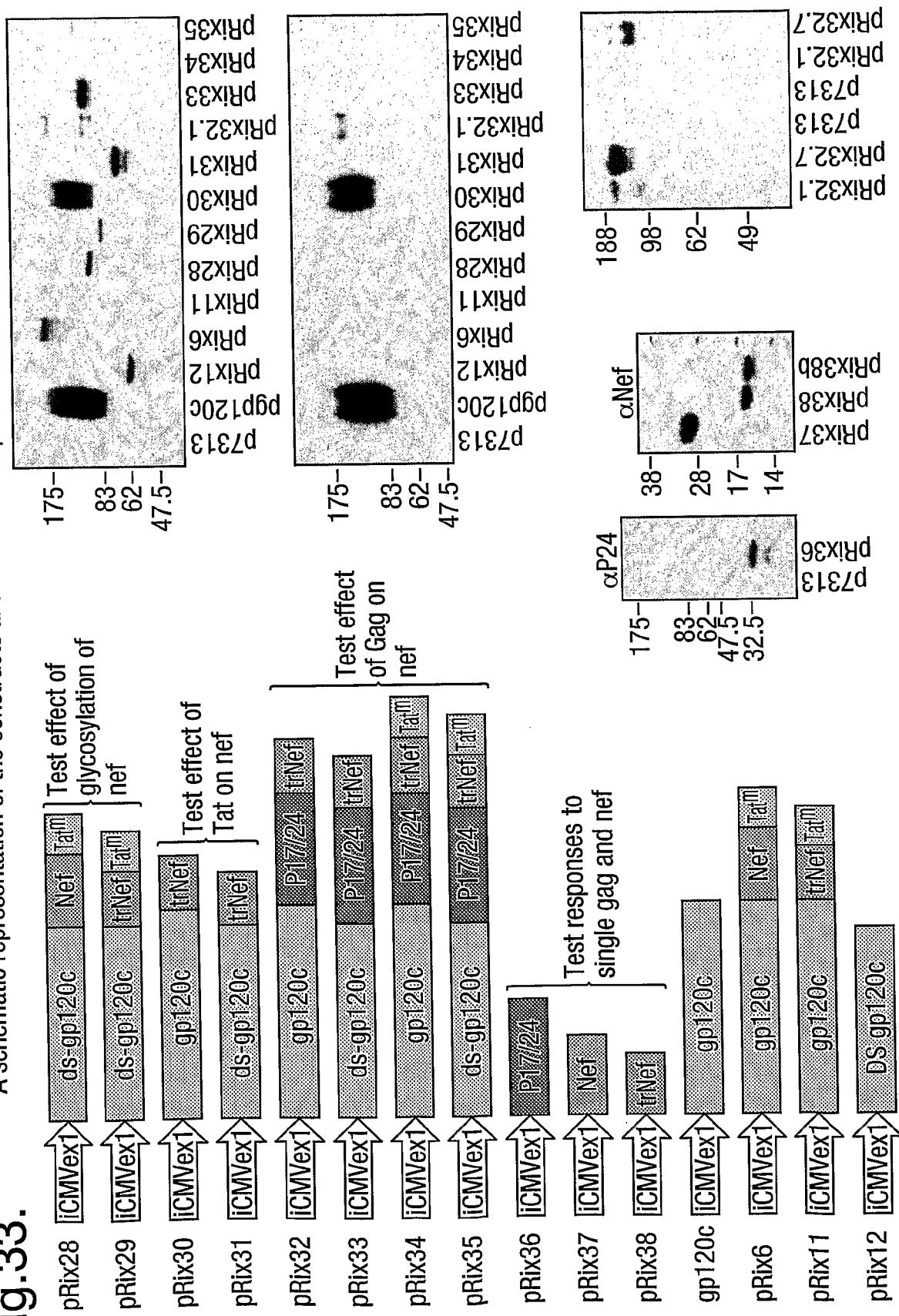


Fig.34.

A Schematic representation of further constructs

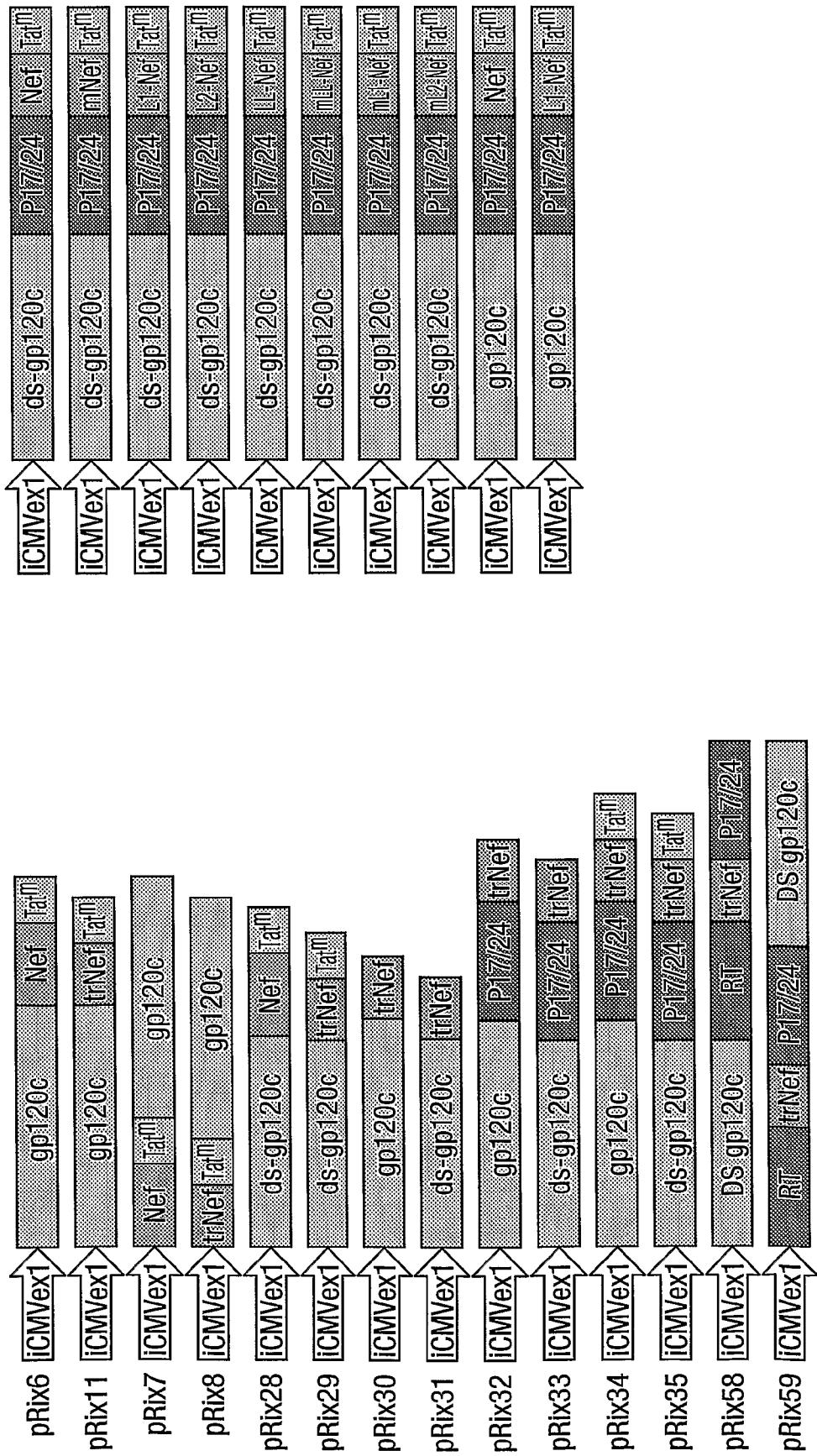
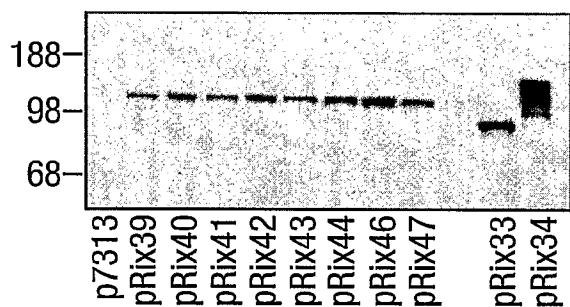
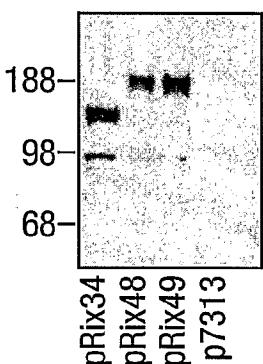


Fig.35.

Expression data (anti-Nef) for dsgp120/Gag/Nef/Tat fusions with mutations in Nef (pRix40-47)



Expression data (anti-Nef) for dsgp120/Gag/Nef/Tat fusions with glycosylated gp120 (pRix48 and pRix49)



Expression data (anti-Nef) for the quadrivalent fusion proteins containing RT, Nef, Gag and dsgp120, compared to expression of the RT,Nef,Gag fusion alone

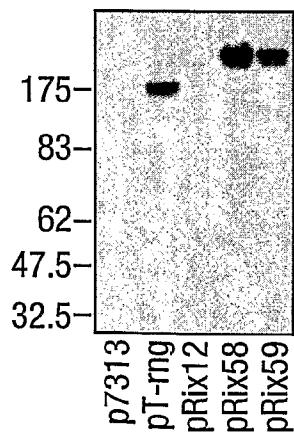
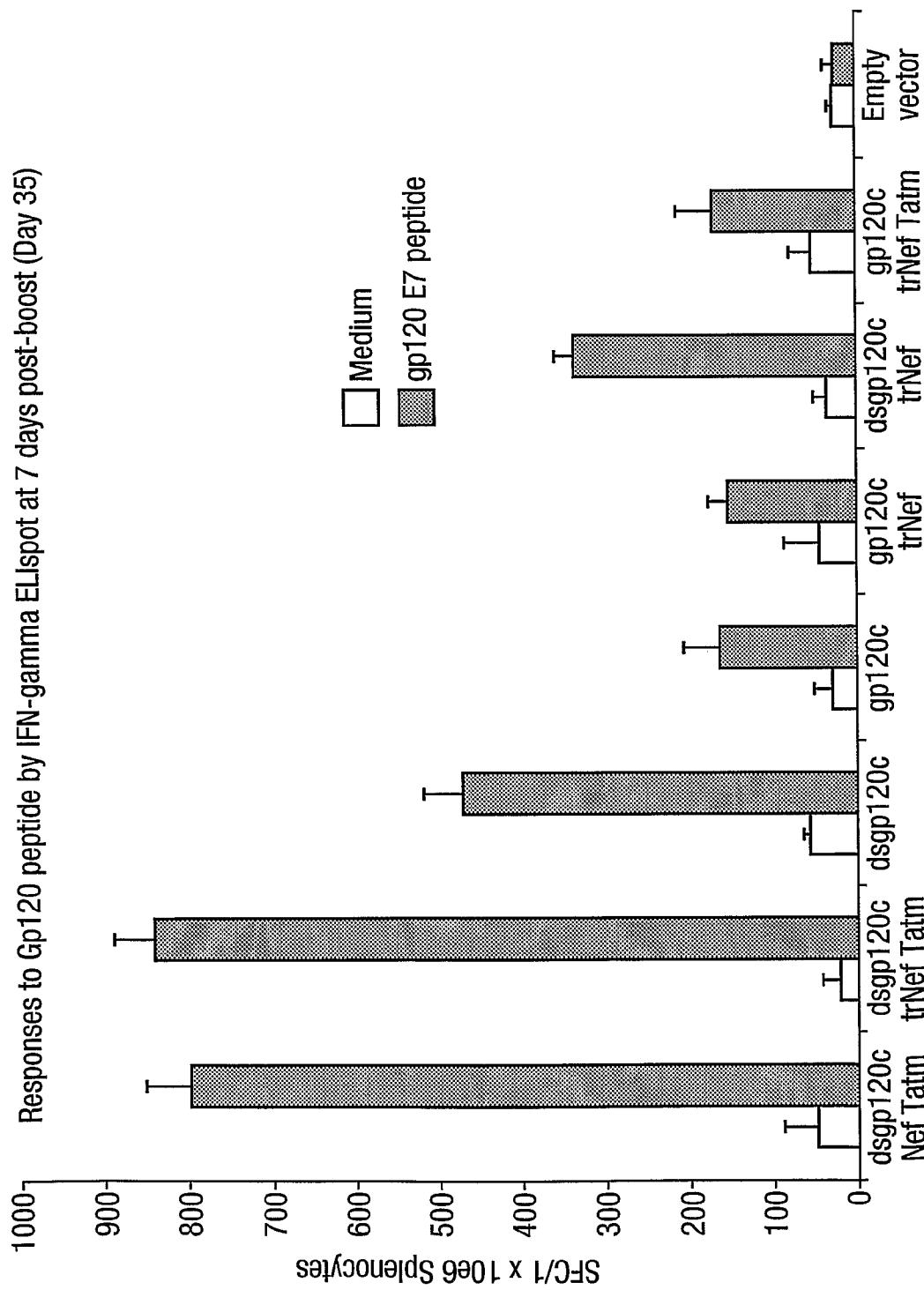
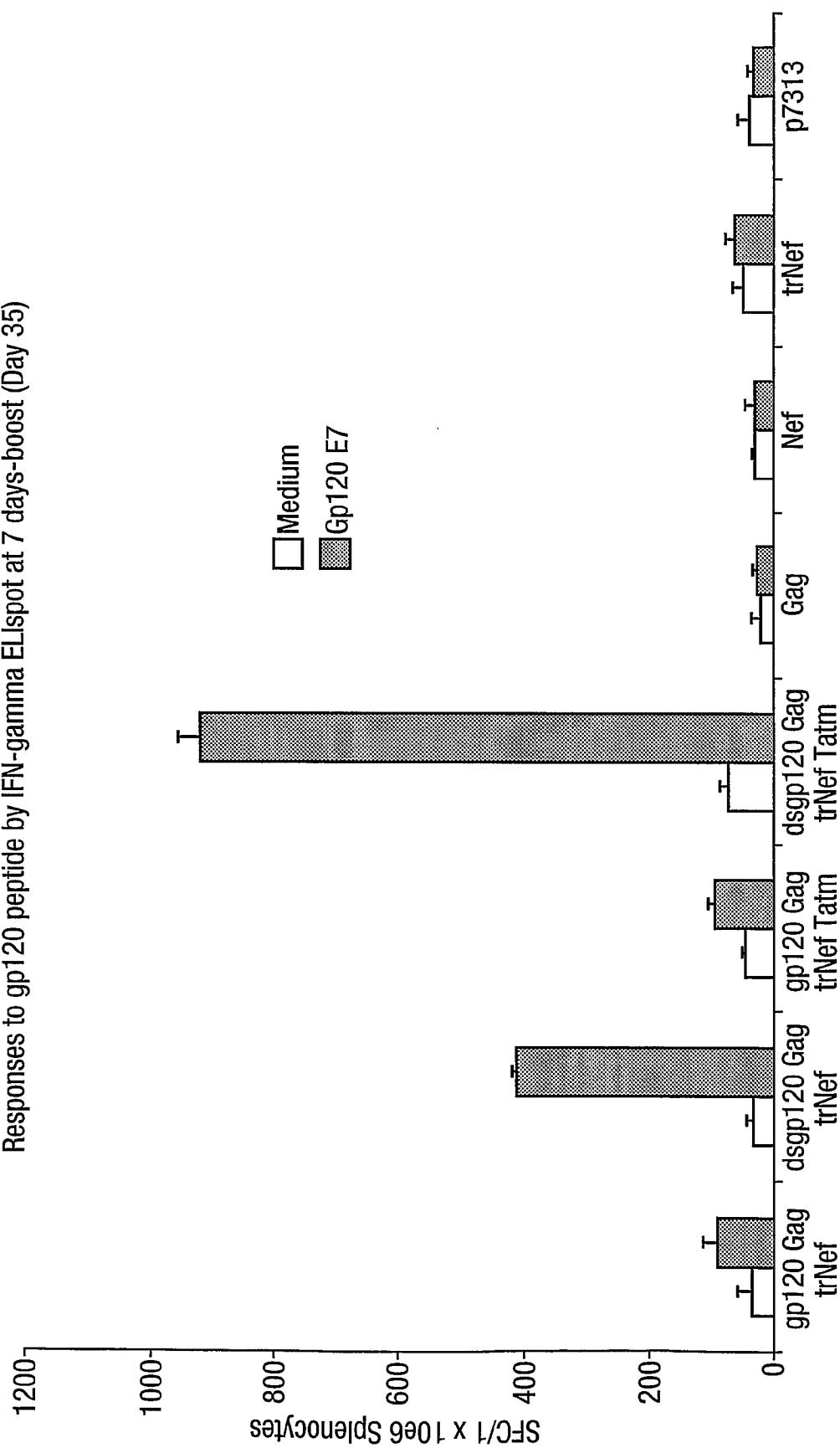


Fig.36.



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Fig. 37.
Responses to gp120 peptide by IFN-gamma ELIspot at 7 days-boost (Day 35)



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Fig. 38.

Response to in vitro restimulation with Gp120, Gag and RT peptides at 7 days post-boost (Day 35) using IFN-gamma Elispot

